Clinical and pathophysiological studies of Tuberculosis

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

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door

Reinout van Crevel
geboren 1 mei 1967 te Rotterdam

Promotor:	Prof. Dr. Jos W.M. van der Meer
Co-promotor:	Dr. Ron H.H. Nelwan, Universiteit van Indonesië
Manuscript commissie:	Prof. Dr. C. van Herwaarden Prof. Dr. P. Smits Dr. M. Borgdorff, KNCV den Haag

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Een neef van Bekker is onlangs gepromoveerd tot Dr. in de Economie op een proefschrift: ‘Pen en gat in de meubelmakerij’.
Ik ben er heen gegaan.
In de vestibule hing een rooster van de colleges. Ik heb niet gezien hoeveel professoren er waren, maar ik denk wel vijftig.
De promovendus zei iets en toen stond er een jong mensch op, een eindje voor me en sprak nog al lang. Ik keek naar z’n ooren. Daarna sprak de promovendus ook nog al lang. Ik kon het niet verstaan en als ik ’t had verstaan had ik ’t niet kunnen volgen. Aan de rechterkant van den promovendus stond een jongmensch, zoo maar, en aan z’n linkerkant zat er een, zoo maar.
De jongeman die zoo lang gesproken had sprak weer en de promovendus gaf weer antwoord. Daarna spraken nog twee van de professoren, een zelfs twee maal en de promovendus antwoordde telkens. Het is wonderlijk hoeveel iemand weten kan over niets.

uit:: Alma Mater (1947) van Nescio

Voor mijn vader Hans

CONTENTS

- clinical and microbiological studies -

Chapter 1

Introduction

Introduction

Tuberculosis (TB), an infection caused by the bacterium Mycobacterium tuberculosis, remains a major and growing health problem throughout the world. In 1997, an estimated eight million people, mostly in their productive age, developed active TB worldwide [1]. In the same year almost two million people died of TB, accounting for one quarter of avoidable adult deaths in developing countries. The growing incidence of TB has renewed the interest of policy makers, clinicians and scientists, who may approach the problem form different angles. We may distinguish a pathophysiological and an operational approach.

The pathophysiological approach is aimed at understanding the pathogenesis of TB. The lifetime risk of developing active TB (in the absence of HIV) after infection is only 5-10%. In addition, among patients with active TB, disease localisation and severity is highly variable. The interindividual differences in outcome after infection with M. tuberculosis are determined by factors related to the microorganism as well as to the human host (**Figure 1**). Increased knowledge about such factors and their role in susceptibility to, and course of TB may contribute to the development of more effective vaccines and new therapeutic strategies.

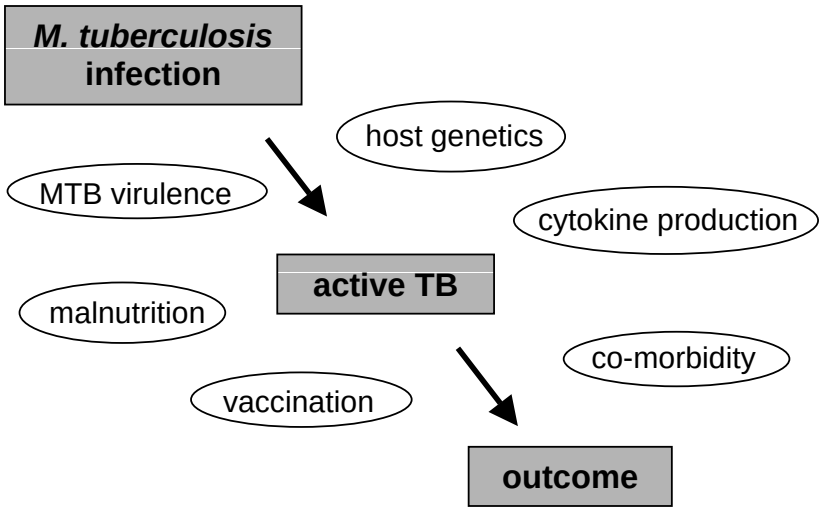

Figure 1. A pathophysiological approach to TB

The operational approach has a very different starting point. Management of TB is complicated by problems related to diagnosis and treatment (**Figure 2**). Such problems may differ between countries and health settings. Clinical, bacteriological and other (e.g. logistic) research may provide (partial) solutions to such problems. This will improve patient care and may reduce the burden of TB.

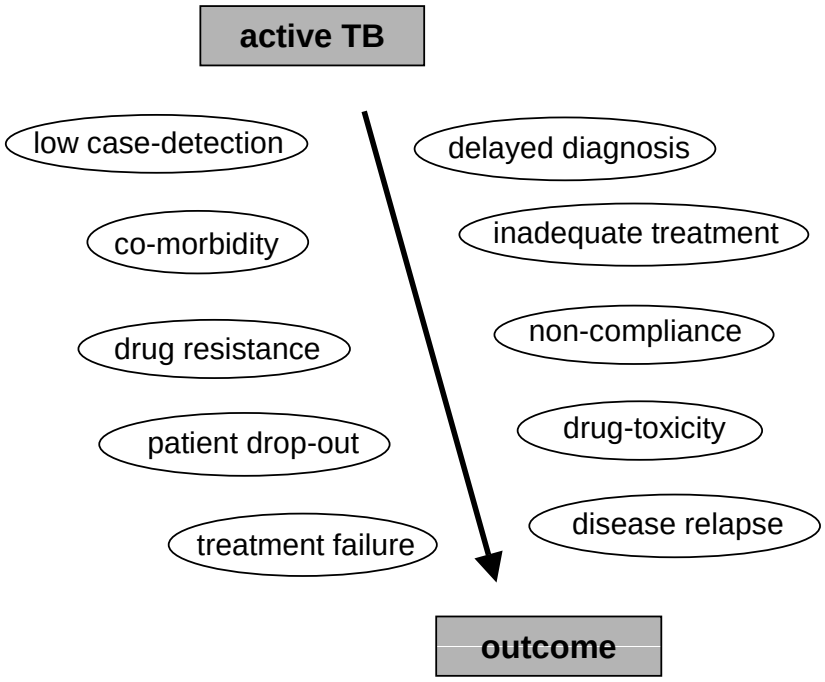

Figure 2. An operational approach to TB.

Outline

The first half of this thesis (chapters 2-6) was written from a pathophysiological point of view and includes investigations into host defense in TB. The second half (chapters 7-11) uses an operational approach and consists of bacteriological and clinical studies in TB. Much of the research from both parts (chapters 4,6,8-11) was conducted in Indonesia.

Traditionally, our acquired T-cell immunity is thought responsible for protective immunity in TB. Recent immunological and genetic studies support the long-standing notion that innate host defense mechanisms also contribute to protection. In chapter 2 the current knowledge of the innate host defense in TB is reviewed. As is clear from this review, cytokines play an important role in the regulation of our immunological response to M. tuberculosis. Chapters 3-5 are specifically related to cytokine production in TB. Cytokine production in humans can be measured in vivo, in the circulation or at the tissue level, and ex vivo, usually by stimulation of isolated white blood cells. In chapter 3 we ask the question whether whole blood cultures can be used as a simple alternative for assessment of the cytokine response in TB patients. Chapter 4 addresses a popular concept in mycobacterial infections: the Th1-Th2 paradigm. Cell-mediated immunity is considered to be responsible for eradication of mycobacteria, but also for damage of host tissues. In this respect, animal models suggest that Th1-type cytokines are protective, and Th2-type cytokines are harmful. In this chapter the question is asked whether an imbalance between production of gamma-interferon (a Th1-type cytokine) and interleukin-4 (a Th2-type cytokine) exists in human TB, and whether this causes or aggrevates tissue damage. Cytokines are important for protection in TB, but they also account for unwanted effects of the host response such as fever, cachexia and tissue necrosis. In chapter 5 the question is asked whether pentoxifylline and thalidomide, two drugs that have been used in attempts to reduce these detrimental effects of the host response in mycobacterial infections, show synergistic effects for in-vitro modulation of cytokine production.

Severe weight loss (wasting) is a prominent feature of TB, and is probably one of the determinants of disease severity and outcome. However, our understanding of the exact interplay between nutritional status and host defense mechanisms in TB remains incomplete. In chapter 6, the role of leptin, an adipocyte product involved in regulation of body weight and cellular immunity, is investigated in TB patients in Indonesia. The question is asked whether leptin may contribute to TB-associated wasting itself, or to its effects on host response and disease outcome in TB.

The part of this thesis dealing with operational research includes bacteriological and clinical studies. Chapter 7 explores a diagnostic pitfall which may be encountered in patients with suspected TB. Under a microscope, Mycobacterium tuberculosis, which causes TB, cannot be distinguished from nontuberculous (atypical) mycobacteria, many of which are harmless. In this chapter, the question is asked what the significance and clinical implications are of finding nontuberculous mycobacteria in patients with suspected TB in a Dutch TB-clinic.

Chapters 8-11 are based on studies conducted in Indonesia. This country has the third highest TB case load worldwide, but little is known about clinical aspects of this epidemic such as the quality of diagnosis and treatment, HIV-infection and drug

resistance of M. tuberculosis isolates. In Chapter 8 some of these questions are addressed in a prospective cohort study in a TB-clinic in a poor area of Jakarta. This first evaluation raised further questions (chapters 9-11). In countries endemic for TB, diagnostic performance may suffer from the burden of patients. In chapter 9, the question is asked whether a simple intervention can increase the yield of sputum microscopy for diagnosis of TB in this setting. In chapter 10 the question is asked whether M. tuberculosis “Beijing” genotype, which is emerging worldwide, and which is associated with multidrug resistant TB, is present among Indonesian TB patients. Chapters 11 focusses on therapy. Plasma concentrations of antituberculous drugs may be relevant for drug toxicity and cure rates. The question is asked whether patients under TB-treatment in Jakarta have therapeutic concentrations of rifampicin, a key drug for treatment of TB. Chapter 12 contains a summary of the answers to these questions and a general discussion of this thesis.

The separation of host response and clinical practice in this thesis is somewhat artifical. During my PhD-project, the above mentioned pathophysiological and operational approach have cross-fertilized and strengthened each other. Cytokine studies in this thesis directly led to the clinical and bacteriological studies conducted. In turn, good clinical practice was needed for the patient-related immunological research. In addition, careful observation of patients, especially in Indonesia, has initiated further pathophysiological research. Therefore, while this thesis is internally diverse and ramified, I hope to be able to convince the reader of its main thrust: the combination of pathophysiological and operational research is natural, and helpful in the battle against TB.

1. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA 1999; 282:677-686.

Chapter 2

Innate Immunity to *Mycobacterium tuberculosis*

Reinout van Crevel [1], Tom H.M. Ottenhoff [2], Jos W.M. van der Meer [1]

[1] Dept of Internal Medicine, University Medical Center Nijmegen, the Netherlands [2] Dept. of Immunohematology and Blood Transfusion, Leiden University Medical Center, The Netherlands

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Abstract

The different manifestations of infection with M. tuberculosis (MTB) reflect the balance between the bacillus and host defense mechanisms. Traditionally, protective immunity to tuberculosis has been ascribed to T-cell mediated immunity with a crucial role for CD4+ T-cells. Recent immunological and genetic studies support the long-standing notion that innate immunity is also relevant in tuberculosis. In this review, emphasis will be on these natural, innate host defense mechanisms, referring to experimental data (e.g. studies in gene-knock out mice), and epidemiological, immunological and genetic studies in human tuberculosis. The first step in the innate host defense is cellular uptake of MTB, which involves different cellular receptors and humoral factors. Toll-like receptors seem to play a crucial role in immune recognition of MTB, which is the next step. The subsequent inflammatory response is regulated by production of pro- and anti-inflammatory cytokines and chemokines. Different natural effector mechanisms for killing of MTB have now been identified. Finally, the innate host response is necessary for induction of adaptive immunity to MTB. These basic mechanisms augment our understanding of disease pathogenesis and clinical course, and will help designing adjunctive treatment strategies.

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Introduction

One-third of the world population is infected with Mycobacterium tuberculosis (MTB), but only 5 to 10% has a lifetime risk of developing active tuberculosis, either within one or two years after infection (primary tuberculosis), or thereafter (postprimary tuberculosis, **Figure 1**). When active tuberculosis develops, disease localization, severity and outcome are highly variable. Miliary tuberculosis, characterized by the hematogenous dissemination of large numbers of mycobacteria throughout the body, is the most serious disease manifestation. On the other end of the clinical spectrum, tuberculous pleuritis is usually self-limiting. Tuberculosis may develop anywhere in the body, but usually presents as pulmonary infection, ranging from mild infiltration to chronic, cavitary and severely destructive disease. The different manifestations of infection with MTB reflect the balance between the bacillus and host defense mechanisms, in which the quality of host defense determines outcome. In this review, emphasis will be on the natural, innate host defense mechanisms to MTB. However, to enable the reader to place those mechanisms in the context of both innate and acquired defense, a complete picture of MTB infection is briefly reviewed first. One should be aware that dissecting innate and acquired host defense mechanisms is an artificial approach. In real life the two components of the host response are complementary and synergistic.

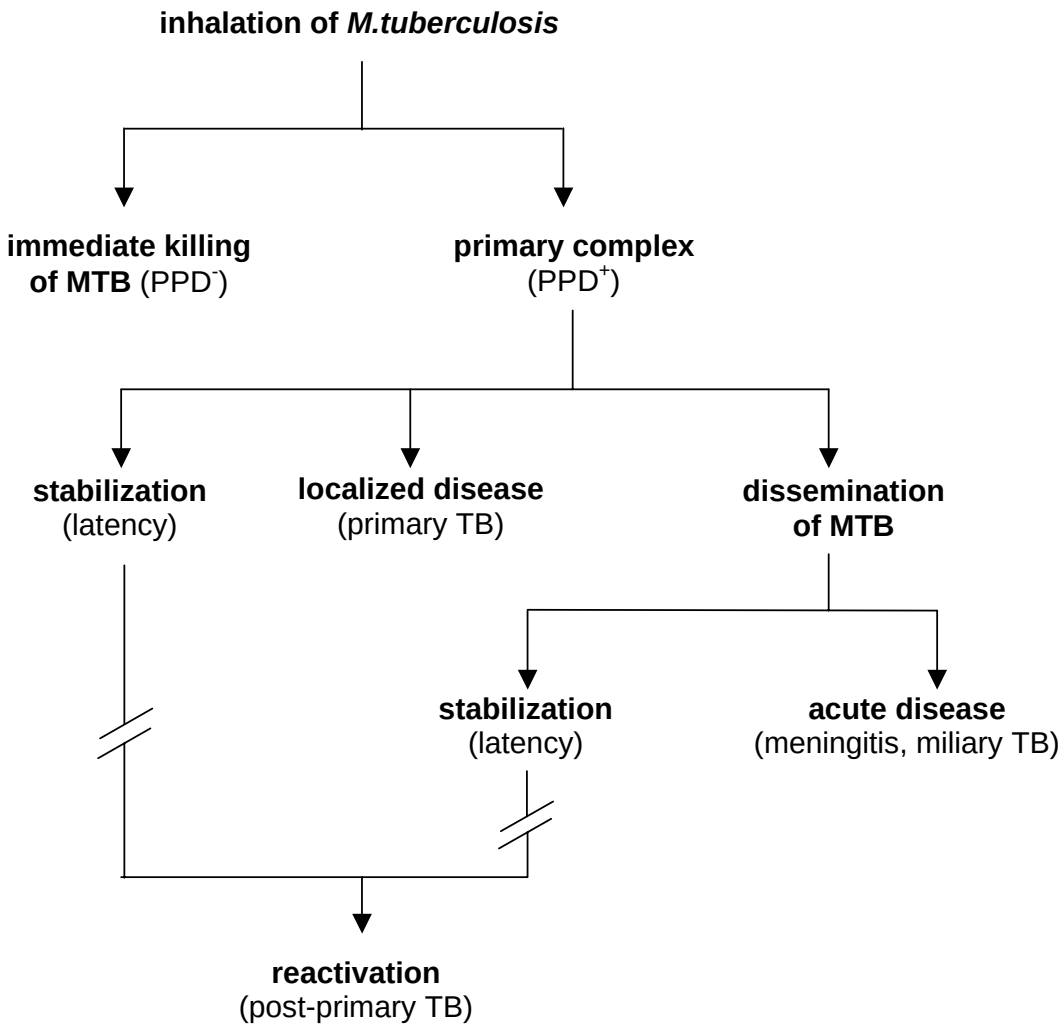

Figure 1.

After inhalation of *M.tuberculosis* (MTB) droplet nuclei, several 'scenarios' may follow. Mycobacteria may be destroyed by alveolar macrophages, in which case no real infection will take place. Alternatively, MTB is not immediately killed: a 'primary complex' consisting of a small infiltrate and a draining lymph node will develop. Small calcifications may be seen on radiographic examination and the PPD skin-test, as a marker of a MTB-specific T-cell response, becomes positive. Most often, infection is stabilized at this point. In a minority of cases active disease now develops (primary TB), either in the lungs or anywhere else after hematogenous dissemination of MTB. Months or years afterwards, usually under conditions of failing immune surveillance, latent infection may reactivate (post-primary TB).

Chronological events in the pathogenesis of tuberculosis

Based on Lurie's fundamental studies in rabbits [1], four stages of pulmonary tuberculosis have been distinguished [2]. The first stage begins with inhalation of tubercle bacilli. Alveolar macrophages (AM) ingest the bacilli and often destroy them. At this stage, the destruction of mycobacteria depends on the intrinsic microbicidal capacity of host phagocytes, and virulence factors of the ingested mycobactera. Mycobacteria that escape the initial intracellular destruction will multiply, and this will lead to disruption of the macrophage. When this happens, blood monocytes and other inflammatory cells are attracted to the lung (second stage). These monocytes will differentiate into macrophages, which again readily ingest but do not destroy the mycobacteria. In this symbiotic stage, mycobacteria grow logarithmically, and blood-derived macrophages accumulate, but little tissue damage occurs. Two to three weeks after infection, T-cell immunity develops with antigen-specific T-lymphocytes that arrive, proliferate within the early lesions or tubercles, and then activate macrophages to kill the intracellular mycobacteria. Subsequent to this phase the early logarithmic bacillary growth stops (third stage). Central solid necrosis in these primary lesions inhibits extracellular growth of mycobacteria. As a result, infection may become stationary or dormant. Disease may progress and hematogenous dissemination may take place after primary infection, as well as months or years afterwards (postprimary tuberculosis), under conditions of failing immune surveillance. Liquified caseous foci provide excellent conditions for extracellular growth of MTB. Cavity formation may lead to rupture of nearby bronchi, allowing the bacilli to spread through the airways to other parts of the lung and the outside environment. In summary, after entry in the human lung, MTB has a series of encounters with different host defense mechanisms. The final outcome of infection with MTB depends on the balance between outgrowth and killing of MTB, and the extent of tissue necrosis, fibrosis and regeneration.

Protection against tuberculosis; acquired T-cell mediated immunity

Elimination of MTB infection mainly depends on the success of the interaction between infected macrophages and T-lymphocytes. Primary as well as acquired immunodeficiencies, especially HIV-infection, have dramatically shown the importance of cellular immunity in tuberculosis. CD4+ T-cells exert their protective effect by the production of cytokines, primarily interferon-γ (IFNγ), after stimulation with mycobacterial antigens. Other T-cell subsets, like CD8+ T-cells, are likely to contribute as well, by secreting cytokines and lysing infected cells [3,4]. The T-cell response is mostly antigen-specific, and attention has focused on the identification of immunodominant antigens, which might be used for the development of effective vaccines [5]. The acquired T-cell response develops in the context of the major histocompatibility complex (MHC), and polymorphism of MHC may contribute to differences in disease susceptibility or outcome [6-8].

Functional diversity of T lymphocytes may also be relevant. In 1986, it was reported that murine helper T (Th) lymphocytes could be divided into two subsets: Th1 clones were characterized by the production of IFNγ, and Th2 clones by the production of interleukin (IL)-4 [9]. Both subsets develop from naive T-cells, whose differentiation is influenced by the environment: IL-12, produced by activated macrophages and dendritic cells, is the principal Th1-inducing cytokine, while IL-4 promotes induction of Th2 cells [10]. More cytokines and different cellular subsets have been included in this Th1-Th2 concept [11], which is thought to be relevant in many disease entities [12]. In mycobacterial infection, Th1-type cytokines seem essential for protective immunity. Indeed, IFNγ gene knockout mice are highly susceptible to MTB [13], and individuals lacking receptors for IFNγ suffer from recurrent, sometimes lethal mycobacterial infections [14-16]. Th2-type cytokines inhibit the in-vitro production of IFNγ [12,17], as well as the activation of macrophages [18], and may therefore weaken host defense[19]. We and others have shown an increase in Th2-type cytokines in tuberculosis patients [20-24]. However, this is not a consistent finding [25-28] and the relevance of the Th1-Th2 concept in disease susceptibility or presentation remains uncertain.

Protection against tuberculosis; evidence for innate immunity

Phagocytic cells play a key role in the initiation and direction of adaptive T-cell immunity by presentation of mycobacterial antigens, and expression of co-stimulatory signals and cytokines. In addition, innate defense mechanisms of phagocytic cells may be important, as highlighted in Lurie's fundamental studies with resistant and susceptible inbred rabbits [1]. Seven days after primary infection through inhalation of tubercle bacilli, the lungs of susceptible rabbits contained 20- to 30-fold more viable mycobacteria than resistant rabbits [2]. Obviously, this difference during early infection cannot be attributed to T-cell immunity. More recently it was found that acquired T-cell immunity in vaccinated mice effectively protects them from disseminated tuberculosis, but does not prevent the initial pulmonary infection [29,30].

In human disease, the same holds true. Acquired T-cell immunity after vaccination with BCG is more effective against disseminated infection than against pulmonary disease [31]. Similarly, naturally acquired T-cell immunity does not prevent exogenous re-infection of the lung [32]. Thus, local, T-cell independent host defense mechanisms clearly are involved in protection against pulmonary infection. More epidemiological data support a role for innate immunity in human tuberculosis. For example, in racially integrated nursing homes, infection, as measured by tuberculin skin-tests conversion, occurred twice as often in black as in white individuals who were equally exposed to active tuberculosis [33]. Apparently, innate host defense mechanisms at this early stage were less efficient in black residents. In accordance with this, macrophages from Afro-Americans demonstrate a relative

permissiveness for intracellular growth of virulent mycobacteria [34]. Support for the relevance of T-cell independent, intrinsic bactericidal activity of macrophages is also found in genetic studies which have shown associations between tuberculosis and functional gene-polymorphism for various macrophage products [35-38]. There is more evidence, both from clinical and experimental studies, to support the relevance of innate immunity in tuberculosis. In the following paragraphs, the various components and processes that make up the innate host response to MTB will be discussed in more detail.

Phagocytosis of M. Tuberculosis

Alveolar resident macrophages are the primary cell type involved in the initial uptake of MTB. After this first encounter, dendritic cells, and monocyte-derived macrophages also take part in the phagocytic process [39,40]. Endocytosis of MTB involves different receptors on the phagocytic cell (**Figure 2**), which either bind to non-opsonized MTB or recognize opsonins on the surface of MTB. As an example of the latter mechanism, mycobacteria can invade host macrophages after opsonisation with complement factor C3, followed by binding and uptake through complement receptors (CR) 1, CR3 and CR4 [41-43]. The relative importance of the various receptors for complement factor C3 is apparent from experiments in vitro, in which in the absence of CR3, phagocytosis of MTB by human macrophages and monocytes is reduced by approximately 70 to 80% [41,44]. For opsonisation with C3, the split product C3b should first be generated by activation of the complement system. MTB also utilizes part of the classical pathway of complement activation by direct binding to C2a, even in the absence of C4b; in this way the C3b necessary for binding to CR1 is formed [45]. This mechanism facilitates mycobacterial uptake in environments low in opsonins such as the lung. Nevertheless, non-opsonized MTB can bind directly to CR3 [46] and CR4 [47]. However, the best-characterised receptor for non-opsonin-mediated phagocytosis of MTB is the macrophage mannose receptor (MR), which recognises terminal mannose residues on mycobacteria [41,48]. When uptake by CRs and MR is blocked, macrophages may also internalize MTB through the type A scavenger receptor [49]. Fcγ-receptors, which facilitate phagocytosis of particles coated with antibodies of the IgG class, seem to play little role in tuberculosis [50].

Enhanced binding of MTB to epithelial cells or alveolar macrophages may represent a risk factor for developing clinical tuberculosis. Collectins, a structurally related group of proteins including surfactant proteins, mannose binding lectins and C1q, seem to be important in this respect. Surfactant protein A (Sp-A) facilitates the uptake of MTB [51], either through binding to the macrophages [52], type II pneumocytes [53,54], or neutrophils [55]. Interestingly, it has been reported that HIV-infected individuals have increased levels of Sp-A in the lungs, and this results in a

threefold greater attachment of MTB to alveolar macrophages [56]. In contrast, another surfactant protein, Sp-D, has been found to block the uptake of pathogenic strains of MTB in macrophages [57]. It may therefore be hypothesized that the relative concentrations of different surfactant proteins correlate with the risk of infection.

Another member of the collectin family, the plasma factor mannose binding lectin (MBL), may also be involved in the uptake of mycobacteria by phagocytic cells. MBL recognises carbohydrate configurations on a wide variety of pathogens [58], and induces phagocytosis directly through a yet undefined receptor, or indirectly by activation of the complement system [59]. Genetic polymorphisms of the MBL-gene account for significant variability of serum MBL concentrations in different populations [60]. One study has reported elevated serum concentrations of MBL in TB patients [61], and genetic polymorphisms associated with increased production of MBL have been reported to be a relative disadvantage in mycobacterial infections [62].

Although MTB has a tropism for phagocytic cells, it may also interact with non-professional phagocytic cells, such as alveolar epithelial cells [53]. This binding may involve fibronectin, a glycoprotein found in plasma and on the outer surface of many cell types [63]. Similar to M. leprae [64], MTB may bind to epithelial cells since the bacterium produces and secretes the 30-31 kDa antigen 85 complex, a fibronectin-binding protein family [65]. In addition, a 28 kDa heparin-binding adhesin, produced by MTB, will bind to sulfated glycoconjugates on host cells [66].

Thus, there are multiple mechanisms for the uptake of MTB, involving a number of different host cell receptors. Most of these interactions have been demonstrated in vitro and their relative importance in vivo remains to be shown. Distinct routes of entry of MTB may lead to differences in signal transduction, immune activation and intracellular survival of MTB. For example, Fcγ-receptor mediated phagocytosis is directly linked to an inflammatory response, and binding to CR is not [43]. Survival of MTB after binding to CR1 is better than after binding to CR3 or CR4 [67]. Also, phagocytosis of Sp-A opsonised mycobacteria by alveolar macrophages suppresses reactive nitrogen intermediates [51], one of the putative effector mechanisms involved in the killing of mycobacteria [68-70]. Likewise, virulent strains of MTB are phagocytosed through macrophage MR, while attenuated strains are not [41], suggesting that this route of entrance is advantageous to the mycobacterium. Indeed, phagocytosis through MR does not trigger O2- production [71], and MTB exerts an antiinflammtory signal through MR [72]. In vivo, the possible role of these events in immune evasion by MTB remains to be determined. Of interest, strong linkage was found with several markers on chromosome 10p13 [73], where the MR gene is located, in a recent genome scan of 245 families with leprosy in India [74].

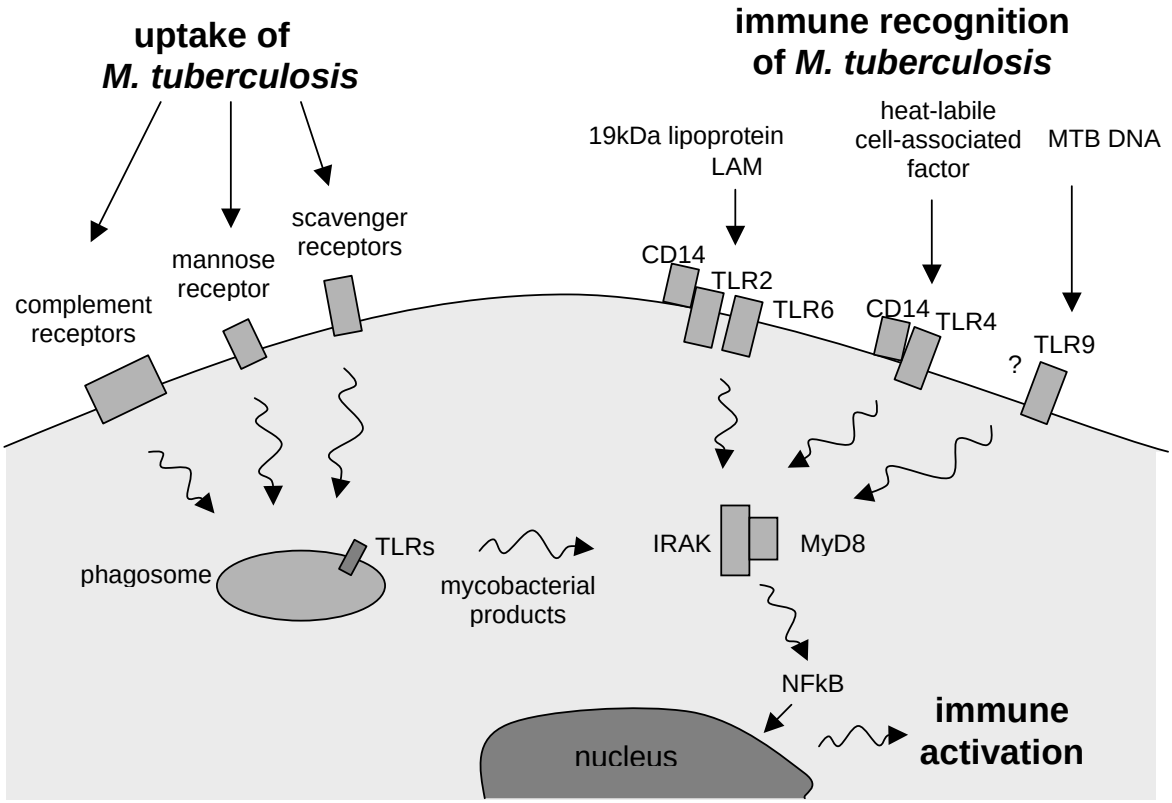

Figure 2.

Phagocytosis and immune recognition of M. tuberculosis

Various receptors have been identified for phagocyosis of *M. tuberculosis* (MTB) by macrophaes and dendritic cells: complement receptors are primarily responsible for uptake of opsonized MTB; mannose receptors and scavenger receptors for uptake of non-opsonized MTB. Toll-like receptors (TLRs) play a central role in immune recognition of MTB. In the context of CD14, TLR2 binds lipoarabinomannan (LAM), TLR4 binds to mycobacterial lipoproteins, and possibly, TLR9 binds to MTB DNA. After binding to TLRs, common signalling pathways lead to cell-activation and cytokine production. TLRs are not only expressed at the cell surface, but also in phagosomes, Therefore, immune activation may occur with or without phagocytosis. On the other hand, phagocytosis alone probably does not lead to immune activation without the involvement of TLRs.

IRAK = IL-1receptor associated kinase; MyD88 = myeloid differentiation protein 88; NFkB = nuclear factor kappa beta

Recognition of M. Tuberculosis; the role of toll-like receptors

Besides phagocytosis, recognition of MTB or mycobacterial products is a crucial step in an effective host response. Immune recognition of the major mycobacterial cell wall component, lipoarabinomannan (LAM), appears to resemble that of gram-negative bacterial lipopolysaccharide (LPS) [75]. Several circulating factors and receptors are involved. Plasma LPS-binding protein (LBP) enhances macrophage responses to LPS and LAM by transferring these microbial products to the cell surface receptor CD14 [76]. Similarly, soluble CD14 confers responsiveness to both LAM and LPS in CD14-negative cells [77]. Of interest, serum concentrations of CD14 and LBP were elevated in patients with active tuberculosis [78].

Toll-like receptors (TLRs) are phylogenetically conserved mediators of innate immunity which are essential for microbial recognition on macrophages and dendritic cells [79-81]. Members of the TLR family are transmembrane proteins containing repeated leucin-rich motifs in their extracellular domains, similar to other pattern-recognizing proteins of the innate immune system. The cytoplasmic domain of TLR is homologous to the signaling domain of IL-1 receptor, and links to IRAK (IL-1 receptor associated kinase), a serine kinase that activates transcription factors like NFkB to signal the production of cytokines [82]. To date, at least 10 TLRs have been identified; of those TLR2, TLR4 and TLR9 seem responsible for the cellular responses to peptidoglycan and bacterial lipopeptides [83], endotoxin of gram-negative bacteria [44], and bacterial DNA [84] respectively.

TLRs are also involved in cellular recognition of mycobacteria (**Figure 2**). Through TLRs, MTB lysate or soluble mycobacterial cell wall-associated lipoproteins induce production of interleukin-12 (IL-12), a strong proinflammatory cytokine [85]. MyD88, a common signaling component that links all TLRs to IRAK [82] was found essential for MTB-induced macrophage activation [86]. A mutation of TLR2 specifically inhibited MTB-induced TNFα production; this inhibition was incomplete, thereby suggesting that beside TLR2, other TLRs may be involved [86]. In a transfection model using Chinese hamster ovary (CHO) cells (that are relatively deficient of TLR), expression of TLR2 or TLR4 conferred responsiveness to both virulent and attenuated MTB [87]. TLR2, and not TLR4, was necessary for signaling of the mycobacterial lipopolysaccharide LAM [86,87] and a 19-kDA MTB lipoprotein [85,88], while an undefined heat-labile cell-associated mycobacterial factor was found to be the ligand for TLR4 [87,89]. Interestingly, mycobacterial infection and proinflammatory cytokines increase surface expression of TLR2 [90]. Besides TLR2 and TLR4, other TLR's may be involved in immune recognition of MTB: heterodimerization of TLR2 with TLR6 or TLR1 is necessary for signal tranduction [91,92], and TLR9 binds CpG dinucleotides in bacterial DNA [84,93].

From several lines of evidence it has become clear that phagocytosis does not lead to immune activation in the absence of functional TLRs (**Figure 2**). Even though

TLR2 is recruited to phagosomes during phagocytosis [94], cytokine production was abrogated by the expression of a mutant TLR2, but particle binding and internalization were unaffected. Furthermore, the expression of CD14 and TLRs did not alter uptake of MTB in in-vitro studies. Apparently, TLRs play an important role in innate recognition of mycobacteria and this also holds for humans. Interestingly, a recent study showed that TLR2 activation directly leads to killing of intracelullar MTB in alveolar macrophages in vitro [95]. It may be anticipated that genetic polymorphism, or perhaps mutations, in the relevant TLR or the downstream signaling proteins will affect the performance of the innate host response to mycobacteria.

Cytokine production driven by M. Tuberculosis

Proinflammatory cytokines

Recognition of MTB by phagocytic cells leads to cell-activation and production of cytokines which in itself induce further activation and cytokine production in a complex process of regulation and cross-regulation. This cytokine network plays a crucial role in the inflammatory response and the outcome of mycobacterial infections (**Figure 3**). Several proinflammatory cytokines will be discussed.

TNFα. Stimulation of monocytes, macrophages [96] and dendritic cells [39] with mycobacteria or mycobacterial products induces the production of tumor necrosis factor-α (TNFα), a prototype proinflammatory cytokine. TNFα plays a key role in granuloma formation [97,98], induces macrophage-activation and has immunoregulatory properties [99,100]. In mice, TNFα is also important for containment of latent infection in granuloma [101]. In TB patients, TNFα production is present at the site of disease [27,102,103]. Systemic spill over of TNFα may account for unwanted inflammatory effects like fever and wasting. Clinical deterioration early in treatment is associated with a selective increase [104], and quick recovery with a rapid decrease of plasma TNFα [105]. To limit the deleterious effects of TNFα [26], s[106]ystemic production of TNFα is downregulated [107-109], and soluble TNFα-receptors which block TNFα-activity are increased [110]. Gene knock out (KO) mice which are unable to make TNFα, [111-113] or the TNFα-receptor p55 [97,114], display an increased susceptibility for mycobacteria. In line with this, the use of potent monoclonal anti-TNFα-antibodies in Crohn's disease and rheumatoid arthritis has been associated with increased reactivation of tuberculosis (including miliary and extrapulmonary disease) [115]. In human tuberculosis, no TNFα-gene mutations have been found and no positive association has yet been established between gene-polymorphism for TNFα and disease susceptibility [6,116].

IL-1β. A second proinflammatory cytokine involved in the host response to MTB is interleukin-1β (IL-1β). Like TNFα, IL-1β is mainly produced by monocytes, macrophages and dendritic cells [117,118]. In TB-patients, IL-1β is expressed in excess [119], and at the site of disease [102,120]. Studies in mice suggest an important role of IL-1β in TB: IL-1α and β double KO mice [121] and IL-1 receptor type I deficient mice (which do not respond to IL-1), display an increased mycobacterial outgrowth, and also defective granuloma formation after infection with MTB [122]. In addition, among 90 Hindu TB-patients in London, haplotypes for IL-1β and IL-1 receptor antagonist (IL-1Ra, a naturally occuring antagonist of IL-1) were unevenly distributed: a “proinflammatory haplotype”, reflected in an increased ratio of IL-1β / IL-1Ra production, was significantly more common in TB pleurisy than in other types of TB [35]. Because TB pleurisy is a usually self-resolving type of primary TB, one may hypothesize that an increased ratio of IL-1β / IL-1Ra protects against a more severe presentation of TB.

IL-6. This cytokine which has both pro- and anti-inflammatory properties [123] is produced early during mycobacterial infection and at the site of infection [102,124,125]. IL-6 may be harmful in mycobacterial infections as it inhibits the production of TNFα and IL-1β [126] and promotes in-vitro growth of M. avium [127]. Other reports support a protective role for IL-6: IL-6 deficient mice display increased susceptibility to infection with MTB [128], which seems related to a deficient production of IFNγ early in the infection, before adaptive T-cell immunity has fully developed [129].

IL-12. IL-12 is a key player in host defense to MTB. IL-12 is produced mainly by phagocytic cells, and phagocytosis of MTB seems necessary for its production [130,131]. IL-12 has a crucial role in the induction of IFNγ production [132]. In TB, IL-12 has been detected in lung infiltrates [103,133], in pleurisy [134], in granulomas [120] and in lymphadenitis [25]. The expression of IL-12 receptors is also increased at the site of disease [135]. The protective role of IL-12 can be inferred from the observation that IL-12 KO mice are highly susceptible to mycobacterial infections [136-138]. In humans suffering from recurrent non-tuberculous mycobacterial infections, deleterious genetic mutations in the genes encoding IL-12p40, and IL-12R have been identified [139-142]. These patients display a reduced capacity to produce IFNγ [143]. Recently, a IL-12R-defect has also been identified in a patient with abdominal TB [144]. Apparently, IL-12 is a regulatory cytokine which connects the innate and adaptive host response to mycobacteria [132,145,146], and which exerts its protective effects mainly through the induction of IFNγ [136].

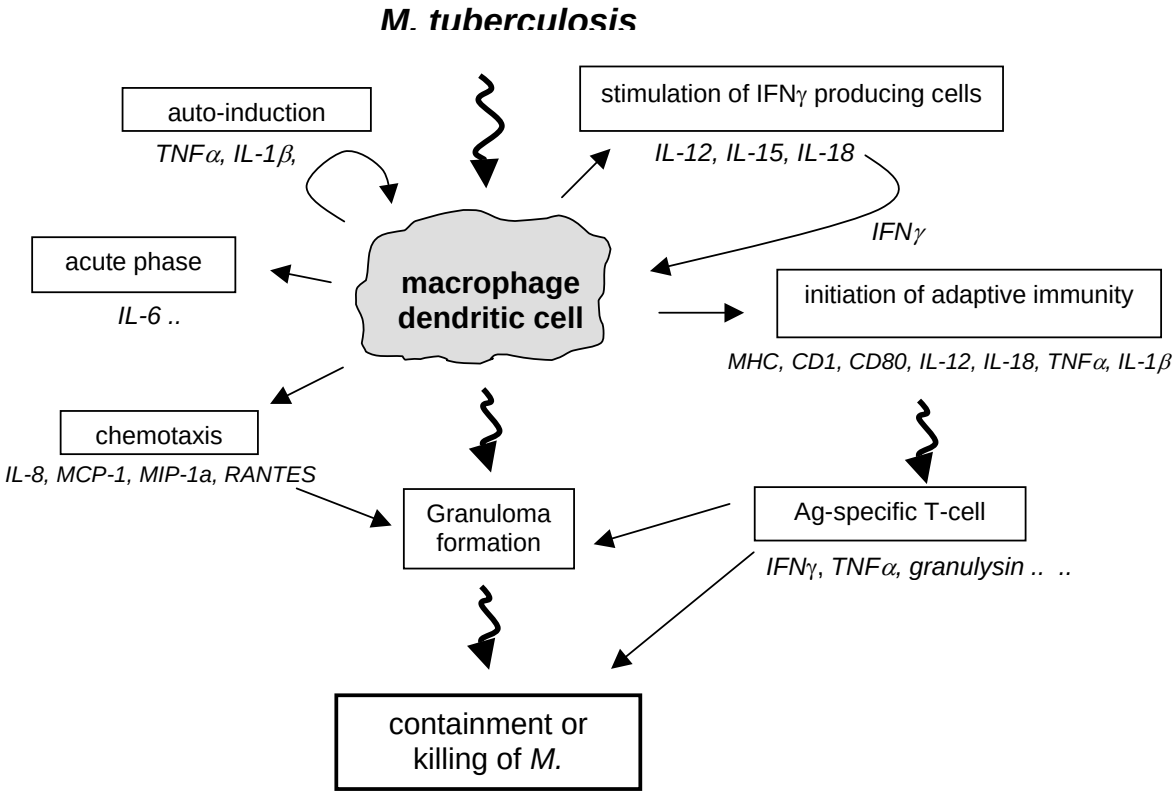

Figure 3.

The inflammatory response of phagocytic cells upon activation with M. tuberculosis.

Immune recognition of *M. tuberculosis* (MTB) by macrophages and dendritic cells is followed by an inflammatory response with a crucial role for cytokine production. Initial events in this cellular response include non-specific host defense mechanisms, which may lead to early killing or containment of infection. In addition, various cellular products, including cytokines and cell-surface markers, are involved in these processes as depicted in the figure (*italics*). Not represented in this picture are the anti-inflammatory cytokines (see text: 'anti-inflammatory cytokines'). Not represented in this figure are the anti-inflammatory cytokines.

IL-18 and IL-15. In addition to IL-12, two cytokines are important in the IFNγ-axis. IL-18, a novel proinflammatory cytokine which shares many features with IL-1 [147], was initially discovered as an IFNγ-inducing factor, synergistic with IL-12 [132]. It has since been found that IL-18 also stimulates the production of other proinflammatory cytokines, chemokines and transcription factors [148,149]. There is evidence for a protective role of IL-18 during mycobacterial infections: IL-18 KO mice are highly susceptible to BCG and MTB [150], and in mice infected with M. leprae, resistance is correlated with a higher expression of IL-18 [151]. IL-18's major effect in this model seems to be the induction of IFNγ. Indeed in TB pleurisy, parallel concentrations of IL-18 and IFNγ were found [152]. Also, MTB-mediated production of IL-18 by PBMC is reduced in tuberculosis patients, and this reduction may be responsible for reduced IFNγ production [152]. IL-15 resembles IL-2 in its biologic activities, stimulating T-cell and NK-cell proliferation and activation [153,154]. Unlike IL-2 however, IL-15 is primarily synthesized by monocytes and macrophages. In leprosy, IL-15 mRNA was found to be expressed more strongly in immunologically resistant tuberculoid leprosy than in unresponsive lepromatous leprosy [155]. As far as we know, no report has been published yet about IL-15 in tuberculosis.

IFNγ. The protective role of IFNγ in tuberculosis is well established [16], primarily in the context of antigen-specific T-cell immunity [5]. Mycobacterial antigen-specific IFNγ-production in vitro can be used as a surrogate marker of infection with MTB [156]. In principal, naive (tuberculin skin-test negative) individuals do not show PPD-stimulated IFNγ production in vitro [156]. However, in both PPD-positive and PPD-negative individuals, MTB-infected monocytes stimulate lymphocytes for the in-vitro production of IFNγ [107]. We found that PPD (consisting of mycobacterial proteins) selectively induces IFNγ production in PPD-positive individuals, while MTB sonicate, which contains mycobacterial polyglycans and phospholipids, non-selectively induced IFNγ production in PPD-positive and PPD-negative individuals alike (van Crevel et al, 2000, unpublished data). This MTB sonicate stimulates production of monocyte derived cytokines like TNFα and IL-1β [157]. These, as well as IL-12 and IL-18, may act as co-stimuli for antigen-independent IFNγ production [132,158,159].

Which cells are responsible for this non-specific production of IFNγ? Firstly, before adaptive T-cell immunity has fully developed, natural killer (NK) cells may be the main producers of IFNγ, either in response to IL-12 and IL-18 [160], or directly by exposure to mycobacterial oligodeoxynucleotides [161]. Second, lung macrophages were found to produce IFNγ in MTB-infected mice [138]. This remarkable observation needs confirmation. Third, T-cells bearing limited T-cell receptor diversity including γδ T-cells and CD1-restricted T-cells may produce IFNγ during early infection. T-cells expressing γδ T-cell receptors (γδ T-cells) may directly recognize small mycobacterial proteins [162] and non-protein ligands [163-165], in the absence of antigen presenting molecules. In mice, a single priming with MTB substantially increases the

number of γδ T-cells, but not the number of αβ T-cells (CD4+ and CD8+ T-cells) in draining lymph nodes [162]. In mice infected with MTB, γδ T-cells accumulate at the site of disease [166], and seem necessary for early containment of mycobacterial infections [167,168]. Like γδ T-cells, CD1-restricted T-cells do not react with mycobacterial protein antigens in the context of MHC class I or class II molecules. Instead, these T-cells react with mycobacterial lipid and glycolipid antigens bound to CD1 on antigen presenting cells [169-172]. CD1 molecules have close structural resemblance to MHC class I, but are relatively non-polymorphic. In mycobacterial infections, several different T-cell subsets have been found to interact with CD1, including CD4- CD8- (double negative) T-cells, CD4 + or CD8+ single positive T-cells and γδ T-cells [173-175]. CD1-restricted T-cells display cytotoxic activity and are able to produce IFNγ [176].

Anti-inflammatory cytokines

The proinflammatory response that is initiated by MTB is antagonized by anti-inflammatory mechanisms. Soluble cytokine receptors (e.g. s TNFα-receptors I and II) prevent binding of cytokines to cellular receptors, thereby blocking further signaling. As already mentioned, IL-1β is counteracted by a specific antagonist, IL-1Ra. In addition, three anti-inflammatory cytokines, IL-4, IL-10 and TGFβ, may inhibit the production or the effects of proinflammatory cytokines in tuberculosis.

IL-10. IL-10 is produced by macrophages after phagocytosis of MTB [177], and after binding of mycobacterial LAM [117]. T-lymphocytes, including MTB-reactive T-cells, are also capable of producing IL-10 [178-180]. In patients with tuberculosis, expression of IL-10 mRNA has been demonstrated in circulating mononuclear cells, and at the site of disease in pleural fluid, and alveolar lavage [27,179]. Ex-vivo production of IL-10 was shown to be upregulated in tuberculosis by some investigators [181,182], but this was not found by others [25]. IL-10 antagonizes the proinflammatory cytokine response by downregulation of production of IFNγ, TNFα and IL-12 [182-184]. Since the latter cytokines – as discussed under the previous heading – are essential for protective immunity in tuberculosis, IL-10 would be expected to interfere with host defense against MTB. Indeed, IL-10 transgenic mice with mycobacterial infection develop a larger bacterial burden [185]. In line with this, IL-10 deficient mice showed a lower bacterial burden early after infection in one report [186], albeit normal resistance in two other reports [187,188]. In human tuberculosis, IL-10 production was higher in anergic patients, both before and after successful treatment, suggesting that MTB-induced IL-10 production suppresses an effective immune response [180].

TGFβ. Transforming growth factor-beta (TGFβ) also seems to counteract protective immunity in tuberculosis. Mycobacterial products induce production of TGFβ by monocytes and dendritic cells [189]. Interestingly, LAM from virulent mycobacteria selectively induces TGFβ-production [117]. Like IL-10, TGFβ is

produced in excess during tuberculosis and is expressed at the site of disease [189,190]. TGFβ suppresses cell-mediated immunity: in T-cells, TGFβ inhibits proliferation and IFNγ production; in macrophages it antagonizes antigen presentation, proinflammatory cytokine production and cellular activation [191]. In addition, TGFβ may be involved in tissue damage and fibrosis during tuberculosis, as it promotes the production and deposition of macrophage collagenases [191] and collagen matrix [192]. Naturally occurring inhibitors of TGFβ abrogate the suppressive effects of TGFβ on mononuclear cells from tuberculosis patients, and in macrophages infected with MTB [193]. Within the anti-inflammatory response TGFβ and IL-10 seem to synergize: TGFβ selectively induces IL-10 production, and both cytokines show synergism in the suppression of IFNγ production [194]. TGFβ may also interact with IL-4. Paradoxically, in the presence of both cytokines, T-cells may be directed towards a protective Th1-type profile [195].

IL-4. The deleterious effects of IL-4 in intracellular infections (including tuberculosis) has been ascribed to its suppression of IFNγ production [12,17] and macrophage activation [18,19]. In mice infected with MTB, progressive disease [196] and reactivation of latent infection [197] are both associated with increased production of IL-4. Similarly, over-expression of IL-4 intensified tissue damage in experimental infection [198]. Conversely, inhibition of IL-4 production did not seem to be promote cellular immunity: IL-4 -/- mice displayed normal instead of increased susceptibility to mycobacteria in two studies suggesting that IL-4 may be consequence rather than cause of TB development [187,188]. In contrast, a recent study on IL-4 KO mice showed increased granuloma size and mycobacterial outgrowth after airborn infection [199]. Compared with control mice, production of proinflammatory cytokines was increased in these animals and accompanied by excessive tissue damage. In human TB patients, we and others have detected increased production of IL-4 in TB patients, especially those with cavitary disease [20,22,119,200]. However, this is not a consistent finding [25-28], and it still remains to be determined whether IL-4 causes or merely reflects disease activity in human TB. Thus, the role of IL-4 in TB susceptibility is not yet entirely resolved.

Production of soluble cytokine receptors and anti-inflammatory cytokines may help to regulate the inflammatory response during tuberculosis. An unrestrained proinflammatory response may lead to excessive tissue damage (as in IL-4 KO mice), while a predominance of anti-inflammatory effects may favor outgrowth of MTB. MTB may evade protective immune mechanisms of the host by selective induction of anti-inflammatory cytokines. In addition, individuals genetically predisposed to higher production of these cytokines may display increased innate susceptibility to MTB. To date, such genetic predisposition has not yet been reported in humans.

Chemokines

Chemotactic cytokines (chemokines) are largely responsible for recruitment of inflammatory cells to the site of infection. About 40 chemokines and 16 chemokine receptors have now been identified [201]. A number of chemokines have been investigated in tuberculosis. First, several reports have addressed the role of interleukin-8 (IL-8), which attracts neutrophils, T-lymphocytes and possibly monocytes. Upon phagocytosis of MTB [202], or stimulation with LAM, macrophages produce IL-8 [202,203]. This production is substantially blocked by neutralization of TNFα and IL-1β indicating that IL-8 production is largely under control of these cytokines [202]. Pulmonary epithelial cells also produce IL-8 in response to MTB [204]. In TB patients, IL-8 has been found in broncheoalveolar lavage fluid [205,206], lymph nodes [120], and plasma [109,203]. Patients who died from tuberculosis showed higher concentrations of IL-8 [109]. Interestingly, upon antituberculous treatment, concentrations of IL-8 remain elevated in alveolar lavage fluid [206] and serum [109] for months. This finding is puzzling, first of all because it is unclear what drives such prolonged production. Secondly, because IL-8 is a potent neutrophil attractant and a neutrophilic response is not prominent in established TB.

A second major chemokine is monocyte chemoattractant protein-1 (MCP-1), which is produced by, and acts on monocytes and macrophages. MTB preferentially induces production of MCP-1 by monocytes [207]. In murine models, deficiency of MCP-1 inhibited granuloma formation [208]. Also, C-C chemokine receptor 2 deficient mice, which fail to respond to MCP-1, display reduced granuloma formation and suppressed Th1-type cytokine production [209], and die early after infection with MTB [210]. In alveolar lavage fluid [206], serum [203] and pleural fluid [211] from TB patients, concentrations of MCP-1 were found elevated. A third chemokine is RANTES, which is produced by a wide variety of cells, and which shows promiscuous binding to multiple chemokine receptors. In murine models, expression of RANTES was associated with development of M. bovis induced pulmonary granulomas [212]. In human patients, RANTES has been detected in alveolar lavage fluid [206]. Apart from IL-8, MCP-1 and RANTES, other chemokines may be involved in cell trafficking in tuberculosis [213]. Inhibition of chemokine production may lead to an insufficient local tissue response. However, due to the redundancy of the chemokine system, the contribution of individual chemokines is difficult to evaluate. As far as we are aware, no clear-cut defects of chemokine production have been identified up to now in patients with mycobacterial infectious diseases.

Effector mechanisms for killing of M. Tuberculosis

Macrophages are the main effector cells involved in killing of MTB. To become active against mycobacteria, macrophages need to be activated. In vitro models of

macrophage activation for the killing of MTB seem rather artificial, and therefore the exact conditions for optimal activation remain unknown. However, it is clear that lymphocyte products, mainly IFNγ, and proinflammatory cytokines like TNFα are important. In addition, vitamin D seems involved in macrophage activation.

The active metabolite of vitamin D, 1,25-dihydroxyvitamin D, helps macrophages to suppress growth of MTB [214-216]. Serum concentrations of vitamin D have reported to be lower in tuberculosis patients in some populations [217], but not in others [218]. A recent study among Gujarati Hindus, a mainly vegetarian immigrant population in London, showed that vitamin D deficiency was a risk factor for tuberculosis [36]. When considered in combination with vitamin D deficiency, three polymorfisms of the vitamin D receptor were also associated with disease susceptibility in this population. For another variant of the vitamin D receptor (tt genotype), 6% of tuberculosis patients in The Gambia proved homozygous compared with 12% of control subjects [37], suggesting that this polymorphism protects against active tuberculosis. It should be noted however, that no functional changes, which might affect macrophage activation, have yet been described for any of the vitamin D receptor polymorfisms associated with disease.

Putative mechanisms involved in killing of MTB within the phagolysosomes of activated macrophages include the production of reactive oxygen or nitrogen intermediates. The study of these mechanisms has been hampered by differences between mice (the most important animal model used for mycobacterial infections) and man. However, when we restrict ourselves to data derived from human cells or patients, controversy remains. In vitro, mycobacteria seem resistant to killing by reactive oxygen intermediates (ROI) such as superoxide and hydrogen peroxide [70]. A possible explanation lies in the fact that several mycobacterial products, including sulfatides and LAM, are able to scavenge ROI [219-221]. In vivo, it was found that p47 (phox -/-) mice, which lack a functional p47 unit of NADPH-oxidase needed for superoxide production, suffer from increased early outgrowth of mycobacteria in experimental infection [222]. Therefore, this supports a role for ROI in the killing of MTB. On the other hand, patients with chronic granulomatous disease (CGD), who have defective production of ROI, do not seem to display increased susceptibility to tuberculosis [223].

The role of reactive nitrogen intermediates (RNI) in tuberculosis also remains a matter of debate. In vitro, human alveolar macrophages infected with M. bovis BCG display increased inducible nitric oxide synthase (iNOS) mRNA [69], and inhibition of iNOS is followed by increased bacterial outgrowth [69]. In tuberculosis patients, alveolar macrophages show increased production of iNOS as well [224]. However, whether iNOS gene expression leads to in vivo NO production remains uncertain as in humans post-translational modification of iNOS may be necessary for functional activity [225]. Therefore, the exact contribution of RNI in human tuberculosis remains to be elucidated.

Sustained intracellular growth of MTB may depend on its ability to avoid destruction by lysosomal enzymes, ROI and RNI. When phagocytosed by macrophages, bacteria typically enter specialized phagosomes that undergo progressive acidification followed by fusion with lysosomes. However, MTB delays or inhibits fusion of phagosomes and lysosomes [50,226]. In addition, MTB prevents phagosomal maturation and acidification of phagosomes, thereby blocking the digestive activity of acidic hydrolases [227,228].

Nramp1, which encodes for natural-resistance-associated macrophage protein (Nramp), is an interesting gene involved in macrophage activation and mycobacterial killing [229]. The protein is an integral membrane protein, which belongs to a family of metal ion transporters. These metal ions, particularly Fe2+, are involved in macrophage activation and generation of toxic antimicrobial radicals [230]. Following phagocytosis, Nramp1 becomes part of the phagosome. Nramp1 mutant mice display reduced phagosomal maturation and acidification [231]. Surprisingly, mycobacterial outgrowth is unaffected in these animals [232]. In humans, functional polymorphism in the promotor region of Nramp1, associated with reduced gene expression, was found to be associated with susceptibility to tuberculosis in studies from West Africa [38,233]. Thus, genetic variation in Nramp1 may affect the outcome of infection with MTB. However, to prove the significance of this gene in human tuberculosis further epidemiological and mechanistic studies are needed.

Apoptosis may constitute another effector mechanism for the infected host to limit outgrowth of MTB [234,235]. Apoptosis of phagocytic cells may prevent dissemination of infection. In addition, apoptosis of infected cells reduces viability of intracellular mycobacteria, while necrosis of infected cells does not [236,237]. TNFα is required for induction of apoptosis in response to infection with MTB [235]. Interestingly, pathogenic MTB strains induced significant less host cell apoptosis than related attenuated strains [235]. This difference was explained by selective induction and release of neutralizing soluble TNFα-receptors by pathogenic strains [238]. Release of TNFα-receptors in turn was regulated by IL-10 production [238]. Thus, pathogenic strains of MTB may selectively induce IL-10 leading to decreased TNFα activity and reduced apoptosis of infected cells. Independent of cytokine production, lipoarabinomannan (LAM) may prevent in-vitro apoptosis of MTB-infected cells in a Ca2+ dependent mechanism [239]. In addition, increased expression of Fas-ligand in infected macrophages may also contribute to decreased macrophage apoptosis [240].

We briefly want to mention the role of other cell-types. Although the precise mechanisms remain to be elucidated, human neutrophils may contribute to killing of MTB [241,242]. However, patients with disorders of neutrophil activity do not show increased susceptibility to tuberculosis. Of more clinical relevance may be the contribution of cytotoxic T-cells [243]. Of special interest, granules of cytotoxic T-cells

and NK-cells contain granulysin, a molecule that directly alters the mycobacterial membrane integrity and thereby kills MTB [244].

Initiation of adaptive immunity to M. Tuberculosis

It is clear that innate and adaptive immunity are closely connected. Macrophages and dendritic cells, the primary cell types involved in the innate immune response to mycobacteria, play a crucial role in the initiation of adaptive immunity. Although an in-depth overview of the mechanisms involved in the adaptive host response to mycobacteria is beyond the scope of this review, we will briefly summarize this subject. In principal, three processes contribute to the initiation of adaptive immunity: antigen presentation, co-stimulation and cytokine production. Patients with active tuberculosis may suffer from anergy or T-cell unresponsiveness [182]. This may be caused by intrinsic defects or dynamic inhibition of one of these three processes.

Antigen presentation. Presentation of mycobacterial antigens by macrophages and dendritic cells involves distinctive mechanisms. First, MHC class II molecules present mycobacterial proteins to antigen-specific CD4+ T-cells. These antigens must be processed in phagolysosomal compartments in professional antigen presenting cells. Second, MHC class I molecules, expressed on all nucleated cells, are able to present mycobacterial proteins to antigen-specific CD8+ T-cells. This mechanism allows for the presentation of cytosolic antigens, which may be important as certain mycobacterial antigens may somehow escape the phagosome [245]. The importance of MHC I mediated antigen presentation has been shown in murine models [246] and tuberculosis patients [4,247]. Third, non-polymorphic MHC-I like type I CD1 (a,b,c) molecules, which are expressed on macrophages and dendritic cells, are able to present mycobacterial lipoproteins to CD1-restricted T-cells. This mechanism of antigen presentation enables the activation of a larger fraction of T-cells at an earlier point in infection, before antigen specificity has developed. A fourth pathway may involve non-polymorphic MHC-Ib molecules [248].

The expression of particular class I and class II MHC alleles in an individual determines the ability of that individual to respond to particular (mycobacterial) antigens and epitopes. Certain allelic human leucocyte antigens (HLA) variants have been associated with tuberculosis [6,249]. HLA-polymorphism may explain the vulnerability of certain isolated populations like Amazonian Indians which have only recently been exposed to tuberculosis [250]. There is a large body of evidence for similar mechanisms in leprosy. The expression of antigen presenting molecules is also a dynamic process, which is regulated by cytokines. While proinflammatory cytokines, primarily IFNγ, stimulate expression of MHC, anti-inflammatory cytokines inhibit its expression. Mycobacteria may modulate the antigen presentation function,

but different results have been obtained in vitro with macrophages and dendritic cells. Mycobacteria may downregulate expression of antigen presenting molecules in macrophages, most likely through the production of anti-inflammatory cytokines [251,252]. On the other hand, MHC expression on dendritic cells is upregulated following MTB infection [39,253].

Co-stimulation. It is well known that antigen presentation only leads to T-cell stimulation in the presence of particular co-stimulatory signals. The most well-known co-stimulatory signals for T-cell stimulation are B-7.1 (CD80) and B-7.2 (CD86). These molecules are expressed on macrophages and dendritic cells and bind to CD28 and to CTLA-4 on T-cells. Interestingly, in vitro infection of monocytes with MTB leads to diminished expression of B-7.1 [254]. On the other hand, MTB infection of dendritic cells induces expression of B7.1, CD40 and ICAM-1 [39]. In the absence of proper co-stimulatory signals, antigen presentation may lead to increased apoptosis of T-cells [255,256].

Cytokine production. Several cytokines produced by activated macrophages and dendritic cells are essential for stimulation of T-lymphocytes. Macrophages and dendritic produce the type-1 cytokines IL-12, IL-18 and IL-23 [257]. In patients with recurrent or fatal nontuberculous mycobacterial infections, functional genetic mutations have been found in the genes encoding IL-12p40 [140,141], IL-12Rβ1 [139,143], IFNγ-receptor 1 [14,15,258] and IFNγ-receptor 2 [259], all of which are involved in IFNγ receptor signaling in macrophages and dendritic cells. Clearly the capacity of these cells to produce or react to Th1-type cytokines is necessary for proper T-cell stimulation (**Figure 4**). In addition, proinflammatory cytokines like IL-1 [260] and TNFα [100] have important T-cell stimulatory properties. Reduced production of type-1 or proinflammatory cytokines may delay or decrease T-cell stimulation and the initiation of antigen specific T-cell immunity. In this respect, the production of anti-inflammatory cytokines may be relevant as well. For instance, in anergic tuberculosis patients it was recently shown that IL-10 production is constitutively present, and that T-cell receptor-mediated stimulation results in defective signal transduction [180]. TGFβ may have a similar antagonistic role [191,193].

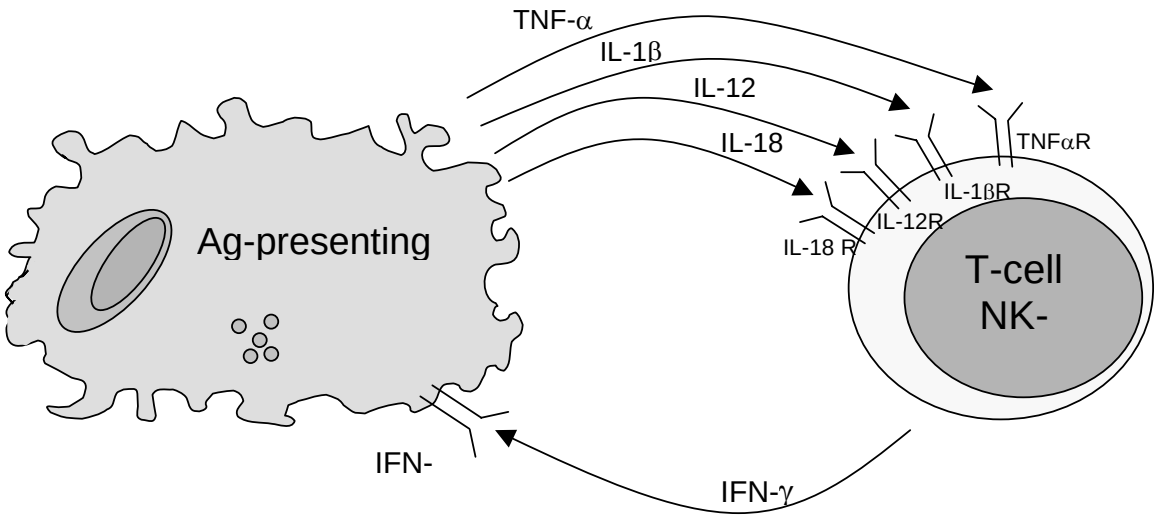

Figure 4.

Cytokines and cytokine receptors involved in type I immunity in tuberculosis.

A major effector mechanism of cell-mediated immunity in tuberculosis is the activation of MTB-infected macrophages by IFNγ. IFNγ is produced by NK-cells and different T-cell subsets, and its production is regulated by TNFα, IL-1β, IL-12, IL-18 and possibly IL-15, all released from activated macrophages and dendritic cells.

Concluding remarks

The interplay between MTB and the human host determines the outcome after infection. With respect to the human host, both innate and adaptive defense mechanisms are involved. After uptake of MTB in alveolar macrophages, several possible scenarios may be envisaged. MTB may be destroyed immediately, in which case no adaptive T-cell response is developed. When infection is established however, a focal nonspecific inflammatory response follows. This response is regulated by a network of pro- and anti-inflammatory cytokines and chemokines. Most of the mediators at this point are derived from macrophages or dendritic cells, but IFNγ has several cellular sources including NK-cells, $\gamma\delta$-T-cells and CD1-restricted T-cells. This initial response determines the local outgrowth of MTB (sometimes dissemination) or containment of infection. Phagocytic cells also play a key role in antigen-presentation and initiation of T-cell immunity that follows. At many stages in the host response, MTB has developed mechanisms to circumvent or antagonize protective immunity.

The interindividual differences in outcome after infection with MTB may in part be explained by the efficiency of various innate host defense mechanisms. Phagocytosis, immune recognition, cytokine production and effector mechanisms may all contribute to innate immunity. In this respect, different gene-polymorphisms have been found which are associated with increased susceptibility and severity of tuberculosis. Some of these polymorphisms are functional, but for many of these no functional (immunologic) changes have been demonstrated yet, and these associations therefore need further confirmation and investigation.

What remains to be determined is to what extent the encounters between MTB and the human host can be modulated. In many settings the most cost-effective way to improve disease outcome is to increase patients access to health care facilities, and to strengthen the quality of diagnosis and antimycobacterial treatment. However, in many parts of the world the spread of multidrug resistant TB seriously threatens the success of antibiotic treatment. Therefore, more effective vaccines and new therapeutic strategies (like immunotherapy) are desperately needed. It is expected that increased understanding of disease pathogenesis will help the design of such adjunctive treatment, which will undoubtedly benefit the outcome of individual patients and limit the spread of MTB around the world.

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Chapter 3

Ex-vivo stimulation of whole blood for cytokine production, applications in the study of tuberculosis.

Reinout van Crevel, Johanna van der Ven-Jongekrijg, Mihai G. Netea, Wiel de Lange*, Bart-Jan Kullberg, Jos W.M. van der Meer,

Dept of Internal Medicine, * Dept of Pulmonary Medicine, University Medical Center Nijmegen, the Netherlands

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Abstract

As a simple method for field studies to assess the cytokine-status of patients with tuberculosis (TB), the use of whole blood instead of isolated cells has advantages, especially since the risk of contamination is minimal. Therefore, cytokine production in whole blood cultures was determined using non-specific and disease-specific stimuli. Heparinized blood from healthy volunteers was either incubated in closed vacutainer tubes or in tissue culture wells after dilution in culture medium. Dose-response and kinetics were investigated for the production of TNFα, IL-1β, IL-1ra, IL-10 and IFNγ. Patients with TB and healthy individuals were examined for IFNγ production in whole blood. In the absence of a stimulus, the production of cytokines is negligible in whole blood cultures. LPS induces the production of TNFα, IL-1β, IL-1ra and IL-10; PHA induces the production of IFNγ and IL-10. Live BCG induces the production of proïnflammatory cytokines, irrespective of tuberculin skin status. In contrast, PPD and MTB-culture filtrate induce production of IFNγ in skin-test positive and not in skin-test negative healthy subjects. Five out of 13 patients with TB had a low antigen-specific IFNγ production, suggestive of a minimal or absent specific T-cell response. For most purposes, cultures in closed vacutainer tubes are optimal. If one wishes to focus on T-cell cytokines or if only small volumes of blood are available, dilution of whole blood in culture medium before incubation in tissue culture wells may be preferable.

Introduction

The host defense in tuberculosis involves both mononuclear phagocytes and lymphocytes [1,2]. Mononuclear phagocytes are able to produce both proïnflammatory cytokines, e.g. tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β) and interleukin-12 (IL-12) and antiinflammatory cytokines, e.g. interleukin-1 receptor antagonist (IL-1ra), interleukin-10 (IL-10) and transforming growth factor-β (TGFβ). Similarly, after stimulation, T-lymphocytes produce so-called Th1-type cytokines, e.g. interferon-γ (IFNγ) and interleukin-2 (IL-2) or Th2-type cytokines, interleukin-4 (IL-4) and interleukin-10 (IL-10) [3]. The delicate balance between pro- and anti-inflammatory cytokines is considered to influence the outcome of disease [4]. For this reason the study of cytokine profiles in patients with tuberculosis may provide valuable information leading to a better understanding of host defense mechanisms against *Mycobacterium tuberculosis*.

Cytokine production in humans can be studied *in vivo*, in the circulation and at the tissue level, and *ex vivo* by stimulation of isolated cells. Concentrations of circulating cytokines are often low or undetectable and do not reflect local cytokine production [5,6]. Measurement of cytokines at the tissue level meets with practical problems in humans and studying ex-vivo cytokine production is associated with other difficulties. In general, isolated white blood cells or cultured cell lines are used. The isolation of cells is cumbersome and cannot be done easily outside the setting of a research laboratory. In addition, the isolation procedure itself may lead to activation of cells [7]. Furthermore, artefacts may arise because of unintentional selection of cells, and because isolated cells, deprived from neighbouring cells and serum components may not retain their in vivo characteristics. In this respect, the study of whole blood ex vivo has both practical and theoretical advantages.

Several systems for ex vivo cytokine production in whole blood have been devised. Broadly, two methods exist. In the first, undiluted whole blood is stimulated and incubated in syringes or closed sampling tubes [8]. In the second method, whole blood is diluted in culture medium before incubation in tissue culture wells [9]. Aiming for a method that permits measurement of both monocyte- and T-cell-derived cytokines, we evaluated ex-vivo cytokine production in undiluted and diluted whole blood. Lipopolysaccharide (LPS), as a stimulus for monocytes, and phytohaem-agglutinin (PHA), which non-specifically stimulates T-lymphocytes, were used, as well as the disease-specific stimuli *Mycobacterium bovis BCG*, *M.tuberculosis* culture filtrate and tuberculin (PPD). Experiments were performed to determine dose-response and kinetics with the blood of healthy volunteers. In addition antigen-specific stimulation of whole blood with PPD was used to try and distinguish tuberculin skin-test positive individuals from control subjects.

Methods

Ex-vivo stimulation of whole blood.

In all experiments, blood samples were taken from healthy individuals between 8 and 9 a.m. unless stated otherwise. Dry vacutainer tubes to which sterile sodium heparin was added and different commercially available glass (Becton and Dickinson, Leiden, the Netherlands) or plastic (Greiner, Leiden, the Netherlands) vacutainer tubes containing lithium heparin, sodium heparin or ammonium heparin were used in a pilot experiment to test for background stimulation by heparin, which appeared to be present to variable degrees. All further experiments were performed using 2-mL glass tubes (Becton and Dickinson) containing lithium heparin. The following stimuli were used; lipopolysaccharide (*E.coli* serotype 055:b5; Sigma, Zwijndrecht, the Netherlands), phytohaemagglutinin (PHA-P, Sigma), bovine tuberculin PPD (ID-DLO, Lelystad, the Netherlands), live *M.bovis BCG* (Danish 1331, RIVM, Bilthoven, the Netherlands) and *MTB* culture-filtrate (a kind gift from Dr.A.H.J. Kolk, Royal Tropical Institute, Amsterdam, the Netherlands). Stimuli were dissolved in phosphate-buffered saline and added shortly after blood sampling with a 1 mL syringe and a 0.6 mm needle through the rubber stopper of the sampling tubes, which remained closed during this procedure. After the indicated times of incubation at 37^0 C, tubes were centrifuged at 2250 x *g* for 10 min. and the supernatants were centrifuged again at 15000 g for 5 min. to obtain platelet-poor plasma. Aliquots were stored at -20^o C until assay.

Alternatively, 200 μl of heparinized blood were diluted 1:4 in culture medium (RPMI 1640 containing 100 IU penicillin/mL) in 24 well tissue culture plates (Costar, Badhoevedorp, the Netherlands). After addition of the above-mentioned stimuli and incubation (37^0 C, 5% CO_2), supernatants were removed and centrifuged at 15000 x g for 5 min before storage at $-20\ ^0$C.

Cytokine measurement.

In all samples the concentration of TNFα, IL-1β and IL-1ra was measured by specific radioimmunoassay (detection levels respectively 40, 40 and 80 pg/mL) as described [10]. Concentrations of IFNγ and IL-10 were measured by ELISA according to the guidelines of the manufacturer (Pelikine, CLB, Amsterdam, the Netherlands).

Dose response and kinetics of ex-vivo cytokine production.

Blood from 5 healthy volunteers was stimulated for ex-vivo cytokine production in both systems with various concentrations of the stimuli and a single incubation period of 24 h. Final concentrations ranged from 1 pg to 100 ng/ mL LPS; 1 to 100 μg/ mL PHA, PPD, MTB-culture filtrate or MTB-sonicate and 10^5 - 10^8 *M.bovis BCG* CFU/ mL. The kinetics of the production of different cytokines in whole blood and diluted whole blood were assessed. Using a single blood sampling tube or tissue culture well

per time point, blood from 4 healthy volunteers was incubated for 1,2,4,8,24,48 and 72 h with final concentrations of 1 ng of LPS /mL, 5 μg of PHA /mL or 10 μg of PPD /mL.

Variation of cytokine response in whole blood; correlation with cell count
Inter- and intra-individual variations of cytokine production in whole blood were assessed. Based on the results of the dose-response and kinetic experiments, standardised conditions for cytokine production were chosen; whole blood was stimulated in vacutainer tubes with 10 ng of LPS/mL or 10 μg PHA/ mL during a six-hour incubation period. For the assessment of inter-individual variation in cytokine production, blood from 20 healthy volunteers was sampled and stimulated between 8.00 and 9.00 a.m. Absolute monocyte and lymphocyte counts and the percentage of T-lymphocytes, as determined by standard flowcytometry, were correlated with cytokine production. Intra-individual variation of ex vivo cytokine production was assessed in ten healthy volunteers. For day-to-day variation of ex vivo cytokine production, whole blood from 10 individuals was sampled on three separate days between 8.00 and 9.00 a.m. For diurnal variation of cytokine production blood from 10 individuals was sampled on one single day at 9 a.m., 12 p.m. and 16 p.m.

Antigen-specific stimulation with PPD; comparison of patients and controls.
IFNγ production after stimulation with 10 μg/mL PPD was assessed in 13 patients with active pulmonary tuberculosis, 13 tuberculin-positive and 13 tuberculin-negative control subjects.

Results

Spontaneous cytokine production in whole blood
Before stimulation as well as after incubation without stimuli, TNFα, IL-1β, IFNγ and IL-10 were not detected. In contrast, IL-1ra was produced in vacutainer tubes without stimulation after 8 h incubation and in tissue culture wells after 4 h. In whole blood from 4 volunteers, the concentrations of spontaneously produced IL-1ra at 24 h were 1.19 ± 0.3 ng/mL in tubes and 1.16 ± 0.46 ng/mL in culture wells compared with 9.35 ± 1.71 ng/mL and 2.95 ± 0.46 ng/mL respectively after 24 h of LPS-stimulation.

Stimulation of whole blood with LPS
LPS is a potent stimulus for the production of TNFα, IL-1β, IL-1ra and IL-10 in whole blood cultures. In undiluted whole blood, production of cytokines continues to rise with increasing concentrations of LPS (**Figure 1**). In contrast, in blood diluted in medium, the production of TNFα, IL-1β and IL-1ra reaches a maximum at 1 ng of LPS/ mL, whereas the production of IL-10 rises with increasing concentrations of

LPS. When corrected for the dilution in culture medium, the production of IL-10 in diluted samples is approximately 15-fold higher than in undiluted blood (**Figure 1**).

Experiments to determine the kinetics of cytokine production in whole blood cultures showed a similar pattern in all subjects. In undiluted whole blood, stimulation with LPS (1 ng/mL) led to maximal concentrations of TNFα and IL-10 at 4 and 8 h, respectively, while IL-1β and IL-1ra continued to rise with time. In contrast, similar stimulation of diluted whole blood in tissue culture wells led to maximal concentrations of TNFα and IL-1β at 8 h, and of IL-10 at 24 h. Using short incubation periods, LPS induced higher concentrations of cytokines in undiluted whole blood. After 4 h of incubation of with LPS (1 ng/mL), the concentration of TNFα and IL-10 in undiluted blood was 3.2 ± 1.2 ng/mL and 168 ± 154 pg/mL respectively, compared with 1.1 ± 0.5 ng of TNFα/mL and 6 ± 1 pg of IL-10/mL in diluted whole blood.

Stimulation of whole blood with PHA

In whole blood, PHA induces a dose-dependent increase of IFNγ, IL-10, IL-1ra and TNFα (**Figure 1**). IL-1β was not detected in PHA-stimulated samples. Using PHA, the production of IL-10 in diluted whole blood was higher than in undiluted blood, whereas the opposite was true for IFNγ, TNFα and IL-1ra. After PHA-stimulation, absolute cytokine concentrations showed high inter-individual variation. However, the patterns of production with time were similar in all subjects tested. Stimulation of undiluted blood in a vacutainer tube with PHA (5 μg/mL) induced maximal concentrations of IFNγ, TNFα and IL-10 at 8 h of incubation, after which concentrations started to fall. Similar stimulation of diluted whole blood in tissue culture wells induced a continued rise in the concentrations of cytokines with time. Short periods of incubation (up to 8 h) led to similar concentrations of IFNγ and IL-10 in the two systems used.

Table 1.

Ex-vivo cytokine production in undiluted whole blood from 20 healthy volunteers

cytokine	before stimulation	LPS-stimulated	PHA-stimulated	no stimulus
TNFα	0.05 ± 0.01	8.44 ± 2.55	nd	0.25 ± 0.22
IL-1β	0.04 ± 0.01	7.7 ± 4.2	nd	0.04 ± 0.01
IL-10	< 5	320 ± 235	127 ± 103	nd
IFNγ	0.008 ± 0.002	nd	7.38 ± 8.94	0.011 ± 0.010
IL-1ra	0.15 ± 0.06	10.05 ± 2.14	nd	0.88 ± 0.20

For ex-vivo cytokine production, whole blood was incubated in closed vacutainer tubes at 37^0 C for 6 h with or without the addition of 10 ng LPS /mL respectively 10 μg PHA /mL. TNFα, IL-1β, IL-1ra and IFNγ (ng/mL) and IL-10 (pg/mL) were measured in culture supernatants. The data are given as mean ± SD. nd = not done.

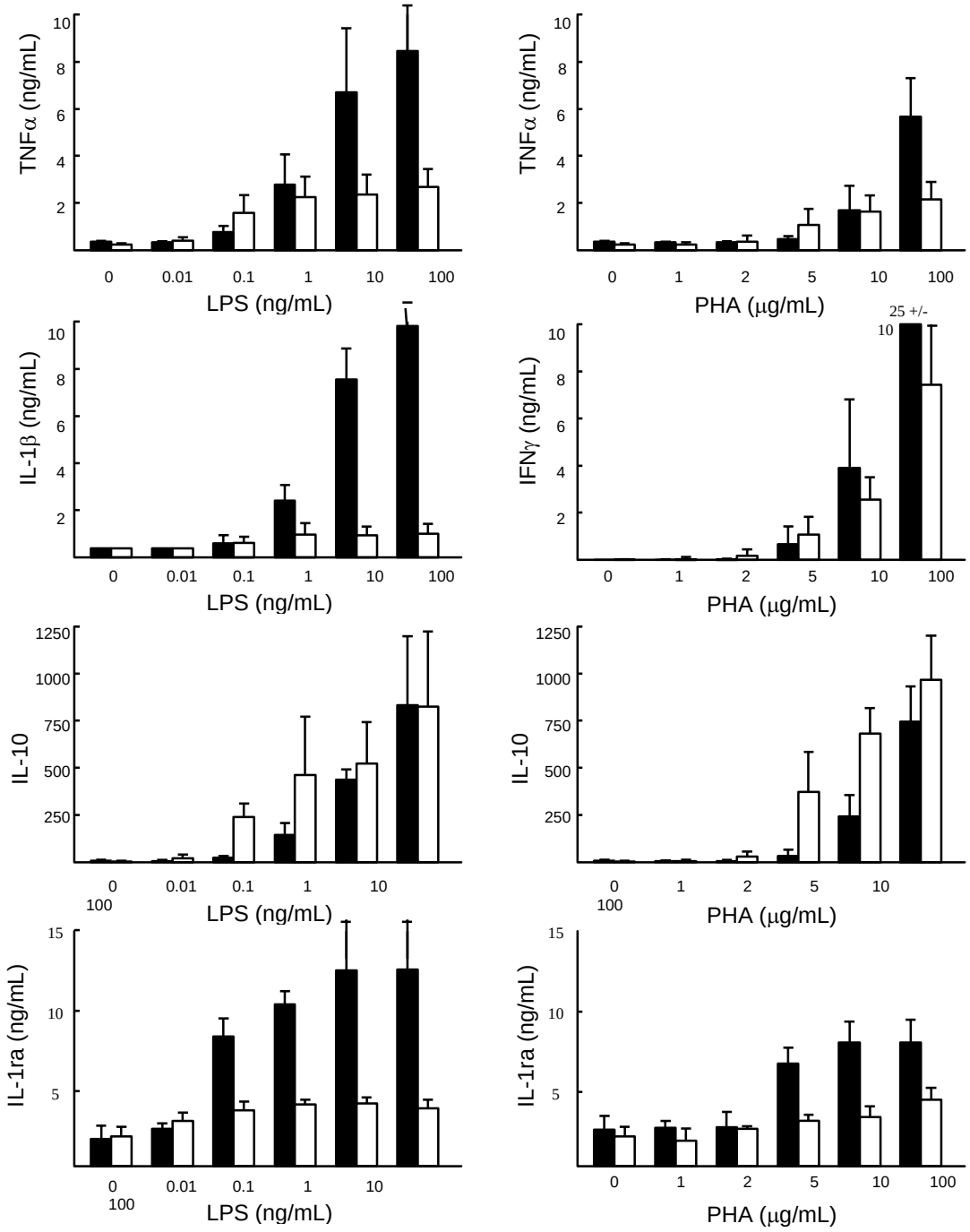

Figure 1. Ex-vivo cytokine production after stimulation with increasing concentrations of LPS or PHA and incubation for 24 h. Cytokine production in vacutainer tubes with undiluted whole blood (■) and in tissue culture wells with whole blood in RPMI (1:4) (□) are shown as the mean (SD) in samples from 5 healthy subjects.

Variation of cytokine response in whole blood; correlation with cell count
Interindividual and intra-individual variation of cytokine production was assessed in twenty healthy subjects using undiluted whole blood. Because stimulation of undiluted blood led to maximal concentrations of TNFα after 4 h of incubation and to maximal concentrations of IL-10 and IFNγ after 8 h, a common incubation period of six h was chosen. The amount of blood drawn into 2 mL vacutainer tubes showed very little variability (2.21 ± 0.078 g). The mean concentrations of TNFα, IL-1β, IL-1ra, IL-10 and IFNγ produced ex-vivo in healthy volunteers are shown in **Table 1**. Inter-individual variation, defined as the standard deviation divided by the mean, ranged from 21% (IL-1ra) to 122% (IFNγ) (**Table 2**). Intra-individual variation of cytokine production, defined as mean variation (SD/mean) in 10 healthy volunteers, is also represented in **Table 2**. Intra-individual variation was smaller than inter-individual variation. Diurnal variation was larger than day-to-day variation for all cytokines tested, except for IFNγ (**Table 2**). Concentrations of IFNγ after stimulation of blood from a single individual were more variable than concentrations of the other cytokines tested. IL-1ra showed very little intra-individual variation. Inter-assay variation was small and not different for the various cytokines measured (**Table 2**). LPS-induced production of TNFα (r^2 = 0.31; *P* = 0.017) and IL-1β (r^2 = 0.44 ; *P* = 0.002), significantly correlated with the absolute number of monocytes in the 20 healthy subjects tested. Production of IL-1ra, IL-10 and IFNγ did not correlate with the absolute T-lymphocyte or monocyte count (data not shown), although a slight and insignificant correlation was found between the number of CD8-positive cells and the production of IFNγ (r^2 = 0.19; *P* = 0.06).

Table 2.
Inter- and intra-individual variation of ex-vivo cytokine production in whole blood

stimulus	cytokine	inter-individual variation (n=20)*	day to day variation (n=10)*	diurnal variation (n=10)*	inter-assay variation (**)	
LPS	TNFα	30 %	12 %	13 %	12,6 %	(37)
	IL-1β	55 %	23 %	32 %	12,1 %	(40)
	IL-10	73 %	17 %	25 %	12,7 %	(17)
	IL-1ra	21 %	5 %	8 %	13,9 %	(37)
PHA	IFNγ	122 %	47 %	32 %	13,6 %	(26)
	IL-10	73 %	16 %	35 %	12,7 %	(17)

To determine the variation in ex-vivo cytokine production, undiluted whole blood was incubated in closed vacutainer tubes at 37^0 C for 6 h after the addition of 10 ng LPS /mL respectively 10 μg PHA /mL. Blood from 20 healthy volunteers was sampled and stimulated on the same day between 8.00 and 9.00 a.m. (inter-individual variation). Three different blood samples from 10 healthy subjects were drawn and stimulated on three separate days between 8.00 and 9.00 a.m (day-to-day variation) and on the same day at 8.00 a.m., noon and 16.00 p.m respectively (diurnal variation). Interindividual variation is SD/mean of 20 individuals (x 100%). Day-to-day and diurnal variation represents the average of variation (SD/mean x 100%) in 10 individuals. * = number of patients; ** = number of assays done.

Antigen-specific stimulation with BCG, PPD and MTB-culture filtrate

(BCG). No detectable concentrations of cytokines were found after stimulation of whole blood with 10^5 or 10^6 live BCG CFU/mL. Using 10^7 or 10^8 BCG CFU/ mL, stimulation of undiluted whole blood led to a dose-dependent production of IFNγ at 24 h of incubation, irrespective of the tuberculin-skin-test of the donor. In four individuals, the mean concentration in plasma after incubation with 10^8 BCG CFU/mL was 4.9 ng/mL, with a maximal concentration of 9.6 ng/mL in one BCG-immunized individual. Small and variable amounts of TNFα (1.55 ± 2.01 ng/mL) were detected, independent of PPD-status. The concentrations of IL-1β and IL-10 were under or just above their limits of detection after BCG-stimulation.

(PPD). PPD induced a dose-dependent increase of IFNγ in the whole blood of two skin-test positive but not of three skin-test negative individuals. In undiluted whole blood, maximal concentrations of 962 and 2000 pg IFNγ /mL were reached after 24 h of incubation with 100μg PPD/mL. In diluted whole blood, maximal concentrations of 136 and 633 pg/mL were found after stimulation with 10 μg PPD/mL blood. Using 100 μg PPD /mL, small quantities of TNFα (mean 0.4 ng/mL) were found, irrespective of the skin-test status. No IL-1β or IL-10 was found after stimulation with PPD. Experiments were performed to determine the kinetics of antigen-specific cytokine production. In three tuberculin-skin-test positive individuals, maximal concentrations of IFNγ (195 -1346 pg/mL) were found after 24 h of incubation of whole blood with PPD, while in diluted whole blood the concentrations continued to rise with longer periods of incubation (106 - 3880 pg/mL at 72 h of incubation).

(MTB-culture filtrate). Irrespective of tuberculin-skin-test status, MTB-culture filtrate induced a dose-dependent increase of TNFα in whole blood from three individuals. In undiluted blood, 100 μg of MTB-culture filtrate/ mL blood led to a maximal concentration of TNFα (mean 8.6 ± 3.0 ng/mL) after 4 h of incubation. In addition, an IFNγ response was found in three tuberculin-skin-test positive individuals. Maximal concentrations (1850-5250 pg/mL) were found after 24 h of incubation of diluted whole blood with 100 μg MTB-culture filtrate / mL.

Antigen-specific stimulation with PPD; comparison of patients and controls.
Antigen-specific stimulation with PPD (10μg/mL) was used to compare IFNγ production in patients with active tuberculosis and healthy controls (**Figure 2**). After a 24-hour incubation period the mean concentration of IFNγ in 13 patients was 769 pg/mL (range 15-3350) compared with 980 pg/mL (range 90-5676) in 13 tuberculin-skin-test-positive individuals and 18 pg/mL (range 4-50) in 13 tuberculin-skin-test negative control subjects. Five out of 13 patients with active tuberculosis had an almost absent antigen-specific production of IFNγ.

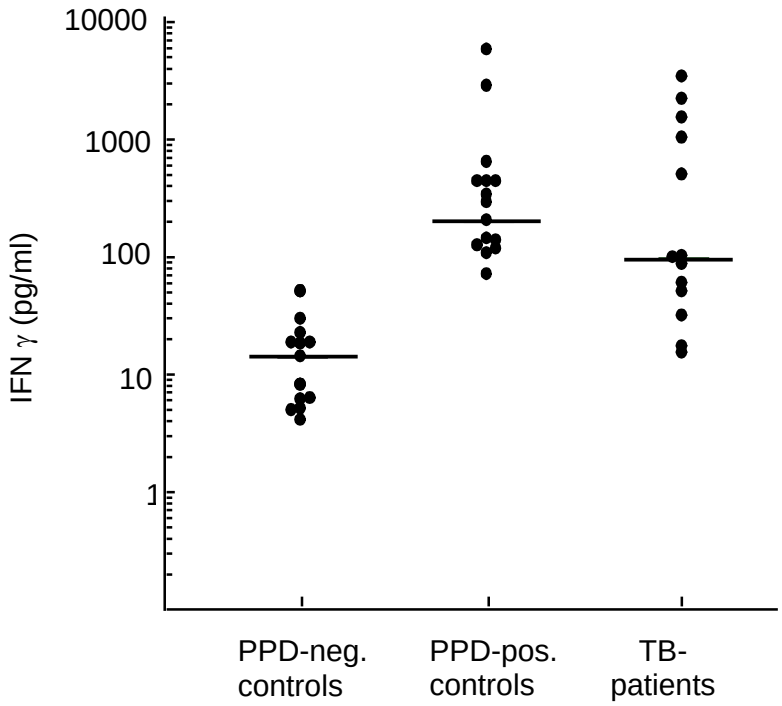

Figure 2
Production of IFNγ (log pg/mL) in whole blood from 13 patients with active pulmonary TB, 13 PPD-positive and 13 PPD-negative control subjects. Undiluted whole blood was stimulated with 10 μg /ml of PPD and incubated for 24 hr. The horizontal bar represents the median for each group.

Discussion

In this paper we describe the development of a clinically useful whole blood cytokine test to assess the ex-vivo production of various cytokines in TB-patients and healthy volunteers. A whole blood culture system using closed vacutainer tubes appeared to be most suitable for field studies, since it is easy to perform with a very small risk of contamination under semi-sterile conditions. Spontaneous production of cytokines in these systems is low, with the exception of IL-1ra. Low-dose LPS induces the production of TNFα, IL-1β and IL-1ra, cytokines that are probably mainly produced by monocytes. PHA induces the production of IFNγ by T-lymphocytes. IL-10, which is produced by both monocytes and lymphocytes, is induced by both LPS and PHA. BCG induces the production of proïnflammatory cytokines. PPD and MTB-culture filtrates induce the production of IFNγ in skin-test positive individuals but not in skin-test negative controls. In addition MTB-culture filtrate induces production of TNFα, irrespective of tuberculin-skin-test status.

To the best of our knowledge, the use of whole blood cultures for cytokine production in research involving TB-patients has only been reported by Friedland et al. [11,12]. No disease specific stimuli were mentioned in their reports and the production of IFNγ, a pivotal cytokine in host defense against MTB, was not reported. In other areas of cytokine research whole blood cultures are increasingly used. However, since its first description in 1982 [9], the use of whole blood cultures for cytokine studies has been poorly standardized. There has been much variation in the stimuli and exact conditions used for incubation [13-16]. The present study was aimed at optimizing a whole blood cytokine test for the study of TB-patients. Ex-vivo cytokine production was assessed in two ways; either by culturing whole blood diluted in medium in tissue culture wells or by stimulating undiluted blood in closed vacutainer tubes. The latter has proven to be a simple and reliable method for ex vivo production of cytokines under primitive conditions [10,17,18]. The dilution of blood and incubation in tissue culture wells facilitates the use of much smaller volumes of blood and longer incubation periods, both of which may be advantageous for the detection of cytokines with a lower production rate (e.g. IL-10). However, stimulation in tissue culture wells requires sterile conditions, and the manipulation of cells may lead to their spontaneous activation. In earlier experiments stationary and agitated samples showed similar results.

The characteristics of whole blood cytokine assays determine both its drawbacks and advantages. Even with optimal standardization many cellular and humoral variables cannot be controlled. Plasma components such as cortisol [19], lipids [20] or soluble cytokine receptors [21] may influence results by enhancing or inhibiting cell-stimulation and inactivating or capturing secreted cytokines. However, all these

effects reflect in vivo conditions, whereas artefacts are created by separating cells and eliminating plasma components. Person to person variation in cytokine production is well known. The extent of variation found in this whole blood assay, clearly not the same for all cytokines tested, determines the significance of differences found between subjects. Diurnal variation underlines the importance of maximal standardization of cytokine assays.

Aiming for a disease-specific whole blood cytokine test to be used in TB-patients, three specific stimuli were evaluated. Live BCG induced the production of proïnflammatory cytokines, irrespective of tuberculin-skin-status. In contrast, PPD and MTB-culture filtrate induced production of IFNγ in skin-test-positive individuals only. In addition MTB-culture filtrate also induced the production of TNFα, irrespective of tuberculin-skin-test status. Circulating memory T-cells probably account for the antigen-specific production of IFNγ in immunized individuals. Antigen-specific stimulation may therefore be used as an alternative to the tuberculin skin test. This approach prevents sensitization and abolishes the need for a return visit. In addition, the sensitivity and specificity of in vitro testing may be higher [22,23]. Apart from its diagnostic value, antigen- specific stimulation may help to assess the prevalence and underlying mechanisms of anergy in latent infection and active tuberculosis [24-27]. In this study, antigen-specific ex-vivo production of IFNγ was very high in some patients but almost absent in others. Longitudinal studies are needed to see if this difference, which is suggestive of specific T-cell unresponsiveness, correlates with a different disease outcomes.

In conclusion, a simple whole blood test has been developed for non-specific and disease specific ex-vivo production of monocyte and T-cell derived cytokines. For most purposes, stimulation and incubation of heparinized blood in closed tubes has major advantages in terms of simplicity, risk of contamination and minimal in vitro manipulation. When focusing on T-cell cytokines, the addition of culture medium may be used to provide a more sensitive test using very small volumes of blood.
The characteristics of whole blood cultures wil permit the study of cytokine networks in both physiological and pathophysiological conditions. Since the outcome of mycobacterial infections is under the influence of pro- and anti-inflammatory cytokines produced by monocytes and T-lymphocytes, the assessment of non-specific and disease-specific cytokine production in whole blood from patients with active tuberculosis or latent infection may increase our knowledge about pathogenesis and help to direct and monitor possible immunotherapy.

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Chapter 4

Increased production of interleukin-4 in tuberculosis is related to the presence of pulmonary cavities.

Reinout van Crevel[1], Elvina Karyadi[4], Frank Preyers[2], Marij Leenders[2], Bart-Jan Kullberg[1], Ronald H.H. Nelwan[3], Jos W.M. van der Meer[1].

[1] Dept. of Medicine, [2] Dept. of Hematology, University Hospital Nijmegen, The Netherlands. [3] Faculty of Medicine, University of Indonesia, Jakarta, Indonesia
[4] SEAMEO-TROPMED, Regional Center for Community Nutrition, University of Indonesia, Jakarta, Indonesia.

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Abstract

In tuberculosis, cellular immunity is considered to be responsible for eradication of infection but also for damage of host tissues. In animal models, the balance between Th1-type cytokines, especially interferon-γ (IFN-γ), and Th2-type cytokines, primarily interleukin-4 (IL-4), seems crucial for these effects. Reports on Th1-type and Th2-type cytokines in human tuberculosis are conflicting, and little is known about their role in tissue damage. To address these issues, flow cytometric assessment of cytokine responses was performed in HIV-seronegative patients with active tuberculosis and healthy controls. Patients and controls showed no significant difference in expression of IFN-γ. However**,** patients demonstrated a striking increase in production of IL-4, in CD4+ as well as CD8+ T-cells. Most remarkably, the expression of IL-4 was especially elevated in patients with cavitary tuberculosis. The Th2-type response with increased production of IL-4 in patients with tuberculosis may antagonize host defense and lead to tissue necrosis.

Introduction

In tuberculosis, cell-mediated immunity (CMI) is responsible for eradication of mycobacteria. The major effector mechanism of CMI is thought to be the activation of infected macrophages by Th1-type cytokines, particularly IFN-γ. The protective effects of Th1-type cytokines may be antagonized by Th2-type cytokines, primarily IL-4 [1]. Like in leprosy the balance between Th1-type and Th2-type cytokine responses in tuberculosis may influence mycobacterial growth as well as immunopathology.

In human tuberculosis, studies on the patterns of T-cell cytokines have met with conflicting results [1-5]. Uncertainty also remains about the cellular source of the various cytokines in tuberculosis. $CD4^+$ T-cells have long been regarded as the major immune regulatory cells. $CD8^+$ T-cells were viewed as CD4-dependent, IFN-γ secreting cytotoxic cells, whose main function was the MHC class I-restricted lysis of infected host cells. Therefore, most studies on T-cell cytokine profiles have concentrated on CD4+ T-cells, although expression of IL-4 has also been demonstrated in $CD8^+$ T-cells in mycobacterial infections.

In atypical mycobacterial infections in man, a Th1-type response is essential [6]. In murine tuberculosis, Th1-type cytokines are necessary for protective immunity [7], while increased production of Th2-type cytokines may be responsible for tissue damage [8,9].

To further evaluate the Th cytokine response in human tuberculosis, to establish the cellular source, as well as the possible role in tissue damage, cytokine production by circulating T-lymphocytes of patients with active tuberculosis with and without cavitation was investigated by flow cytometry and compared to tuberculin-skintest positive controls.

Methods

Subjects

Eighteen HIV-seronegative patients with active pulmonary tuberculosis were included at a University Hospital in Jakarta, Indonesia. Eleven were male (61%) and the average age was 32.8 years (range 16-66). Most patients had extensive pulmonary involvement; 11 had pulmonary cavities, and four had miliary disease. Fourteen tuberculin-skin-test positive healthy individuals of similar age and sex were recruited as controls.

Cell stimulation and immunofluorescent staining.

Intracellular cytokines by circulating T-lymphocytes were detected as described [10], with minor modifications. Briefly, 1 x 10^6 peripheral blood mononuclear cells (PBMC), isolated by density centrifugation, were stimulated with 25 ng/ml phorbol myristic acetate (PMA, Sigma, Zwijndrecht, the Netherlands) and 1 μg/ml ionomycin (Sigma) in the presence of 10 μg/ml brefeldin (Sigma), to inhibit protein secretion. Due to the low number of antigen-specific T-cells in the circulation, stimulation with PPD did not yield a detectable signal (not shown). After 4 hours of incubation cells were fixed and permeabilized using Fix and Perm (Caltag, Burlingame, Ca.) according to the manufacturer's guidelines. CD4 cannot be used for phenotypic subsetting after stimulation since it is heavily down regulated by PMA [11]. CD3-PE-Cy5 and CD8-PE (Dako, Cilostrup, Denmark) were therefore used to define $CD8^+$ T-cells and $CD8^-$ T-cells (mainly $CD4^+$ T-cells), further referred to as $CD4^+$ T-cells. After permeabilization, cells were incubated for 20 minutes with anti-IFN-γ-FITC and/or anti-IL-4-PE (Pharmingen, San Diego, Ca.). PBMC from several patients and control subjects were assayed simultaneously. Interassay variation was less than 15% (not shown).

Flow cytometric analysis

Cells were analyzed for immunofluorescence in a Coulter Epic XL (Coulter, Hialeah, Fl.). $IL\text{-}4^+$ and IFN-γ^+ cells were determined in the $CD4^+$ $CD3^+$ and $CD8^+$ $CD3^+$ cell population in separate measurements (minimum: 50000 cells). Spontaneous expression of cytokines was similarly low in patients and control subjects (mean: 0.75% for IFN-γ and 0.6% for IL-4). For final analysis, residual expression of cytokines was subtracted from expression after maximal stimulation.

Statistical Analysis

Comparisons between groups were performed using Mann-Whitney-U-test and Chi^2-test. *P*-values were two-sided and the level of significance was set at $P < 0.05$.

Results

T-cells from TB-patients do not produce more IFN-γ than controls.
After stimulation of PBMC, IFN-γ was detected in $CD8^+$ and $CD4^+$ T-cells from all patients and controls. Within the subset of $CD4^+$ T-lymphocytes, the percentage of cells staining positive for IFN-γ was almost identical in the two groups, ranging from 7.8-29.9% (mean: 12.7%) in patients and 4.7-21.3% (mean: 11.8%) in controls. Within $CD8^+$ T-cells, expression of IFN-γ was detected in 29.0% (range 10-65%) of stimulated cells from patients, compared with 21.3% (5.7-42%) in controls ($P = .16$).

Both $CD4^+$ and $CD8^+$ T-cells from TB-patients produce more IL-4 than controls.
After stimulation of PBMC, T-lymphocytes also expressed IL-4. Both in $CD4^+$ and in $CD8^+$ T-cells, production of IL-4 was significantly higher in patients with tuberculosis than in healthy controls. The percentage of $CD4^+$ T-cells positive for IL-4 ranged from 1.5-7.3% in patients (mean: 3.9%) and from 0.4–2.4% (mean: 1.4%) in control subjects ($P < .001$). In $CD8^+$ T-cells expression of IL-4 exhibited a wide range in patients (0.4–21.0%; mean 4.7%) and was low or undetectable (range: 0–2.0%; mean 0.7%) in control subjects ($P < .005$). Five patients expressed IL-4 in more than 5% of $CD8^+$ T-cells. Analysis of a second sample of these 5 individuals yielded similarly high expression (6.9-18.7 %).

The ratio of IFN-γ and IL-4 production is decreased in TB-patients.
The ratio of production of IFN-γ to IL-4 in single individuals has been used as a marker for Th1-Th2 balance, with a lower ratio pointing towards a Th2 response [1]. For $CD4^+$ cells, this ratio was much lower in patients than in controls (3.9 *vs.* 9.6; $P < .001$). The same was true for $CD8^+$ T-cells (21.6 *vs.* 36.2; $P < .05$). **Figure 1** depicts the simultaneous detection of IFN-γ and IL-4 in circulating T-cells from a representative patient after in-vitro stimulation of PBMC. Unstimulated cells from this patient demonstrate spontaneous ex-vivo production of IFN-γ in 0.9% of $CD3^+$ cells (**Fig.1A**). Stimulation of cells induces expression of IFN-γ in 10.1% of T-cells (**Fig.1B**). In the same patient, 0.6% of unstimulated T-cells and 13.0% of stimulated T-cells express IL-4. Double staining for IFN-γ and IL-4 in single T-cells was equally low in all individuals examined (<1.2%).

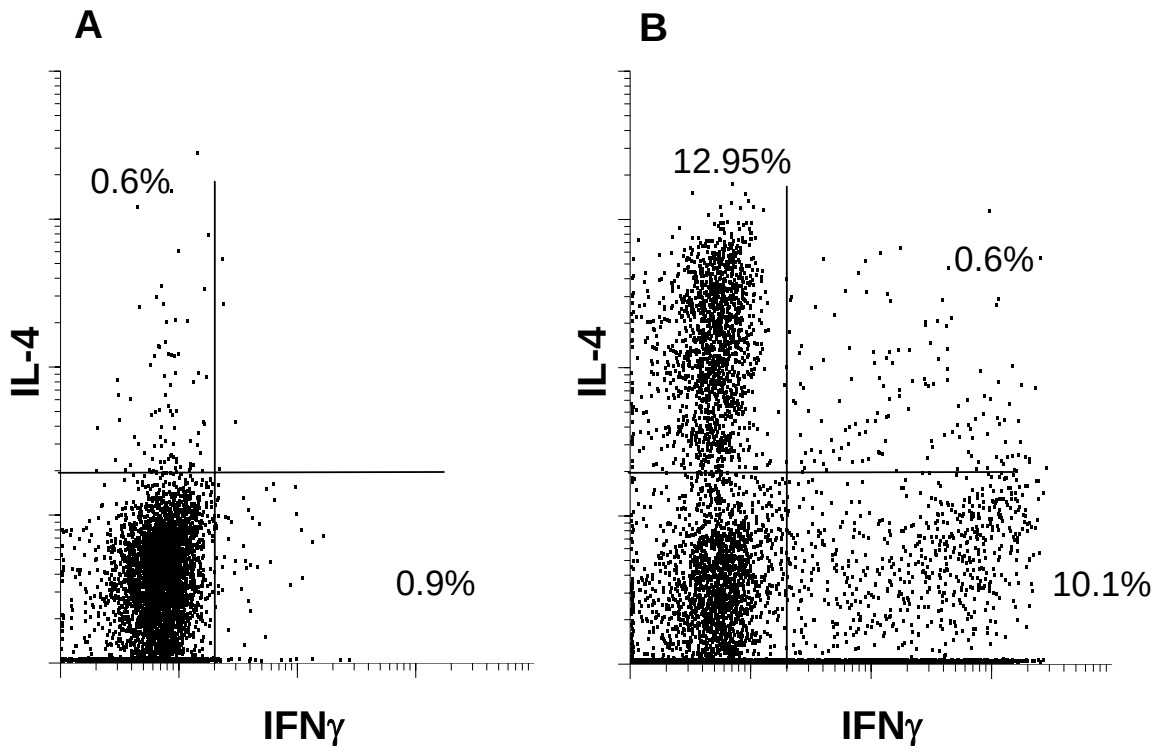

Figure 1

Expression of IL-4 and IFN-γ in circulating T-cells from a patient with cavitary pulmonary tuberculosis. Flow cytometric assessment of cell-specific cytokine response in unstimulated (A) and stimulated (B) PBMC was performed using immunfluorescent antibodies against CD3, IFN-γ and IL-4. Depicted is the % of $CD3^+$ cells expressing IFN-γ or IL-4.

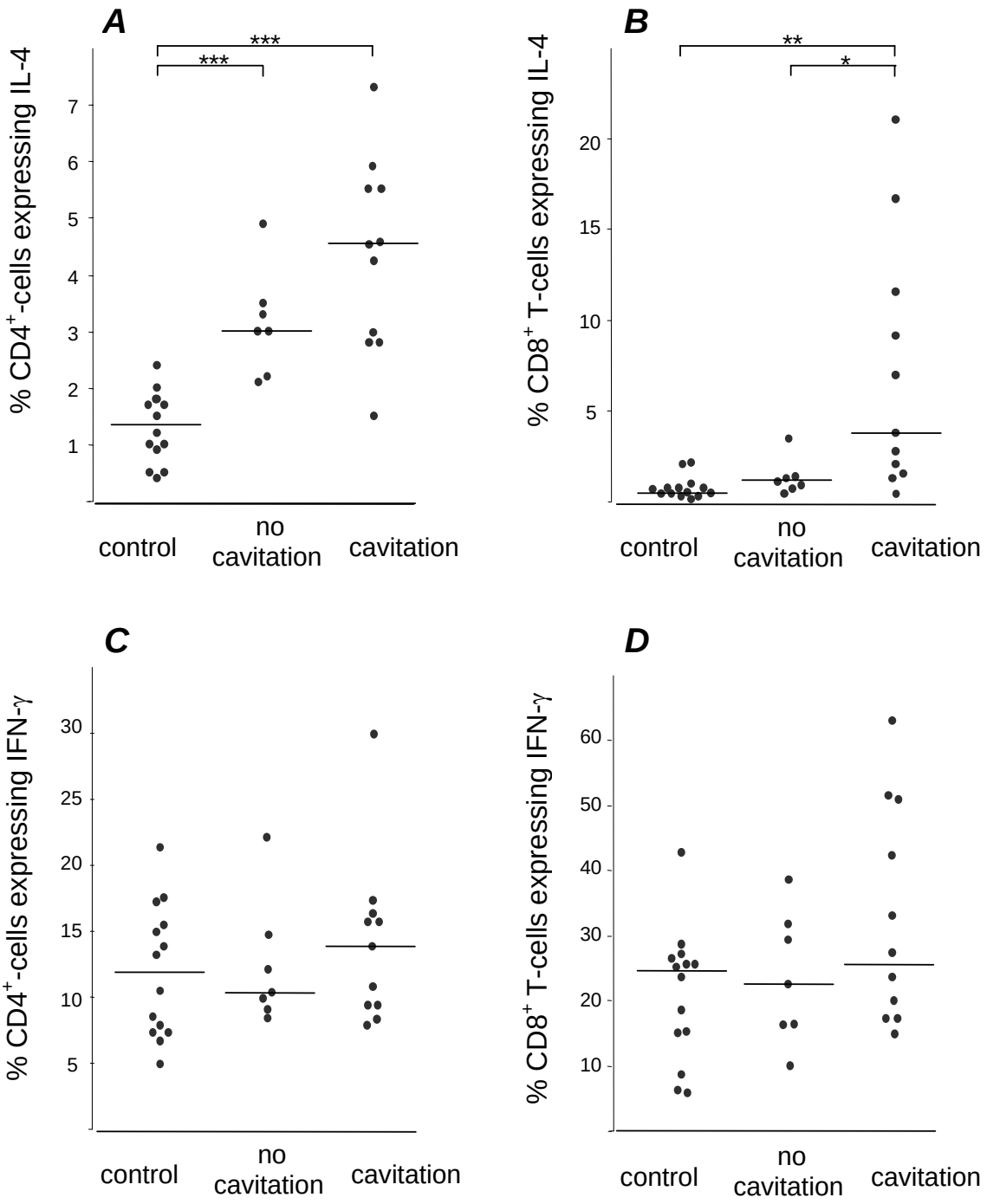

Figure 2

Expression of IL-4 and IFN-γ in TB patients with pulmonary cavities, patients without cavities and PPD-positive healthy controls. Depicted are the % of $CD4^+$ and $CD8^+$ T-cells expressing IL-4 (A, B) and IFN-γ (C, D). Each dot represents one individual. Horizontal bar is median. Significant differences in production of IL-4 were found between groups (Mann-Whitney-U-test; *** $P < 0.0005$; ** $P < 0.001$; * $P < 0.05$). No significant differences in production of IFN-γ were found.

Cavitary tuberculosis is associated with increased IL-4 production

Next, expression of cytokines was related to clinical characteristics. Extent and nature of pulmonary involvement, as exemplified by the presence of cavities on chest X-ray, demonstrated a correlation with production of IL-4, but not with IFN-γ (**Fig 2**). The median number of $CD8^+$ T-cells expressing IL-4 was 3.7% in eleven patients with pulmonary cavities compared with 1.1% in seven patients without cavities ($P < .05$). In contrast, the number of $CD8^+$ T-cells expressing IFN-γ was almost similar in patients with or without pulmonary cavities (27.0% *vs*. 22.4%; P = .22). As a result, the ratio of IFN-γ to IL-4 production in $CD8^+$ T-cells was much lower in cavitary disease (median: 7.4 *vs.* 26.5; $P < .05$). Within the subset of $CD4^+$ cells, expression of IL-4 also tended to be higher in patients with pulmonary cavities (4.5% *vs.* 3.0%; $P = .19$). Expression of IFN-γ and ratio of IFN-γ to IL-4 in $CD4^+$ was similar in both groups. IL-4 was more frequently expressed in $CD8^+$ T-cells than in $CD4^+$ cells in the majority (63%) of patients with pulmonary cavities, while this never occurred in patients without cavities ($P = .01$).

Four patients with miliary disease, all severely ill, exhibited much lower IL-4 expression, especially in $CD8^+$ T-cells. The percentage of $CD8^+$ T-cells expressing IL-4 was 0.8 % in miliary and 7 % in cavitary tuberculosis ($P < .05$), while expression of IFN-γ was not significantly different (27.3% vs. 32%, $P = 0.6$).

Discussion

In this study, we demonstrated that patients with pulmonary tuberculosis exhibit a markedly increased capacity for IL-4 production in circulating T-lymphocytes. Production of IL-4 was not only established in $CD4^+$ T-cells, the primary cytokine-producing cell in the Th1-Th2 concept, but also in $CD8^+$ T-cells. Interestingly, the highest expression of IL-4 in these two cellular subsets occurred in patients with pulmonary cavities, suggesting a role for IL-4 in the development of tissue damage.

It is unclear whether increased production of IL-4 causes, or merely reflects severe disease. With regard to the first notion, IL-4 may reduce killing of mycobacteria by phagocytes through inhibition of IFN-γ production [1]. However, in our study, in-vitro production of IFN-γ was not affected. With regard to the second possibility, severely ill patients with miliary tuberculosis did not demonstrate increased expression of IL-4, suggesting that IL-4 is not just a marker of disease severity as such. Our findings suggest that increased production of IL-4 plays a role in tissue necrosis. The deleterious role of IL-4 in various infectious diseases, including leprosy and leishmaniasis, has been ascribed to its suppression of the protective inflammatory response of Th1-type cytokines [1]. However, IL-4 may also induce or intensify tissue damage; only when primed for production of IL-4, mice infected with saprophytic mycobacteria develop peribronchial and intersititial necrosis when [8]. Likewise, overexpression of IL-4 in PPD-sensitized mice increased the size and cellularity of PPD-induced granulomas [9]. Thus, these reports point to involvement of IL-4 in tissue damage in mycobacterial infections in mice, and our observations extend this conclusion to human tuberculosis.

Another interesting finding is the expression of IL-4 in $CD8^+$ T-cells, which are usually viewed as $CD4^+$-dependent, IFN-γ-secreting cytotoxic cells. Until now, the role of $CD8^+$ T-cells in human tuberculosis remains controversial. Specific $CD8^+$ T-cell clones, which recognize *M. tuberculosis* infected cells and react with high expression of IFN-γ, have been obtained from patients [12]. Other reports have challenged a protective role of $CD8^+$ T-cells. Killing of *M. tuberculosis* by human macrophages is dependent on $CD4^+$-cells, but independent of $CD8^+$-cells [13]. High umbers of $CD8^+$ cells in alveolar lavage of TB-patients correlated with delayed resolution of disease [14]. It is conceivable that $CD8^+$ T-cells exert their counterproductive role through secretion of IL-4.

The Th2-type response found in this study of human tuberculosis is in agreement with previous reports [2,3]. In culture supernatants, IL-4 may not be detectable [4,5], since production of IL-4 splice variants, or binding of secreted IL-4 to soluble or cellular receptors interferes with this assay [15]. To circumvent this problem and to

allow for cell specific analysis of Th status in tuberculosis, we used flow cytometric detection of intracellular cytokine expression, which is unaffected by natural release of cytokine inhibitors or receptors [10].

As protective immunity in tuberculosis depends on a cellular host response, with an important role for $CD4^+$ T-helper cells, the Th1-Th2 concept has long thought to be important in the outcome after infection with *M. tuberculosis*. In agreement with several other intracellular infections, a Th1-type response would be beneficial, while a Th2-type response would be ineffective or even harmful in tuberculosis. An essential role for Th1-type cytokines has been established in murine tuberculosis [7] and in nontuberculous mycobacterial infections in man [6]. Assessment of Th1- and Th2-type cytokine patterns after experimental murine infection has demonstrated high production of IFN-γ in early infection versus increased production of IL-4 during the chronic phase of infection that was characterized by progressive fibrosis and necrosis [8].

The finding of a correlation between a systemic Th2-type cytokine response and tissue necrosis in patients with pulmonary tuberculosis is instrumental in explaining the divergent manifestations of mycobacterial disease in humans. Increased understanding of the role of cytokines in determining the delicate balance between protective immunity and immunopathology in tuberculosis may help the design of successful immunotherapy.

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Chapter 5

Modulation of LPS, PHA and *M. tuberculosis* mediated cytokine production by pentoxifylline and thalidomide.

Reinout van Crevel, Alieke G. Vonk, Mihai G. Netea, Bart-Jan Kullberg, Jos W.M. van der Meer

Dept. of Internal Medicine, University Medical Center Nijmegen, The Netherlands.

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Abstract

Pentoxifylline and thalidomide have been used to downregulate production of TNFα in several disease entities including mycobacterial infections and autoimmune disorders. These drugs inhibit the production of TNFα by different mechanisms, but little is known about possible synergism and modulation of other cytokines. Pentoxifylline and thalidomide inhibited the in-vitro stimulated production of TNFα, IL-1β and IFNγ in blood mononuclear cells. No significant modulation of antiinflammatory cytokines was found. When used together, these agents demonstrated additive inhibition, but no synergism. Modulation of cytokine response was similar when different stimuli were used, including *M.tuberculosis* in tuberculin-positive individuals.
Therefore, the balance between efficacy and toxicity may be more favourable when pentoxifylline and thalidomide are used together instead of either drug alone. Clinical studies are needed to establish this advantage when anti-cytokine strategies are considered.

Introduction

Tumour necrosis factor-α (TNFα) is essential to host immunity, but excessive production of TNFα and other proinflammatory cytokines may have deleterious effects in acute and chronic inflammatory conditions. TNFα activity has been implicated as a central mediator in the pathogenesis of a wide range of human diseases including autoimmune disorders, cancer cachexia and endotoxic shock [1]. In human tuberculosis, clinical deterioration after initiation of therapy is accompanied by an increase in TNFα production [2]. Similarly, effective treatment of leprosy may be followed by the development of erythema nodosum leprosum, a toxic syndrome associated with excess production of TNFα [3,4]. Therefore, TNFα may be an important target for pharmacological intervention.

Pentoxifylline, a phosphodiesterase inhibitor licensed for the treatment of claudication, and thalidomide, which has been used as an antiemetic and sedative drug in the past, both inhibit the production of TNFα [5]. These substances have been examined as immunosuppressive or immunomodulatory agents in a number of disease entities including aphtous ulcera during HIV-infection, Behcet's disease, Still's disease, rheumatoid arthritis and graft versus host disease. With regard to mycobacterial infections, pentoxifylline and thalidomide have been used successfully in erythema nodosum leprosum [3,6]. Similarly, thalidomide accelerated weight gain and inhibited TNFα production [7] in active tuberculosis, while pentoxifylline showed moderate beneficial effects in HIV-seropositive pulmonary tuberculosis [8].

Pentoxifylline inhibits the transcription of TNFα mRNA in vitro [5], while thalidomide lowers the production of TNFα by enhancing TNFα mRNA degredation [9]. Since pentoxifylline and thalidomide have different mechanisms of action, combined therapy may lead to synergism, and thus be advantageous in terms of effectiveness and toxicity. Indeed, in patients with refractory rheumatoid arthritis [10], and in a patient with Still's disease [11], the use of both agents together had a more potent clinical effect than pentoxifylline or thalidomide alone. Synergism of pentoxifylline and thalidomide in the inhibition of LPS-induced production of TNFα by isolated monocytes in vitro was claimed in a previous report [9]. Contradictory reports have been published on the effect of these drugs on antiinflammatory cytokines, which may antagonise the effects of proinflammatory cytokines [12-14].

We investigated the effect of pentoxifylline and thalidomide, alone and in various combinations, on proinflammatory cytokines, primarily TNFα, interleukin-1β (IL-1β) and interferon-γ (IFNγ), and on antiinflammatory cytokines like IL-1-receptor antagonist (IL-1ra) and interleukin-10 (IL-10), using in-vitro stimulation of human PBMC with different stimuli.

Methods

Stimulation of human peripheral blood mononuclear cells (PBMC)
Blood from 8 healthy individuals was used in these experiments. In experiments using antigen-specific stimulation with *M. tuberculosis*, blood from 4 tuberculin-positive individuals was used. Isolation of peripheral blood mononuclear cells (PBMC) was performed as described [15], with minor modifications. Briefly, venous blood was drawn in to 10 mL tubes containing 0.2 mg of EDTA (Monoject, 's-Hertogenbosch, the Netherlands). The PBMC fraction was obtained by density centrifugation of blood (diluted 1:1 in pyrogen free saline) over Ficoll-Paque (Pharmacia Biotech AB, Sweden). PBMC were washed twice in saline, and resuspended in culture medium (RPMI 1640 Dutch modification, ICN Biomedicals, Inc., Costa Mesa) supplemented with heat inactivated human serum 5%, gentamicine 1%, L-glutamine 1% and pyruvate 1%). The cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, The Netherlands) and the number was adjusted to $5x10^6$ cells/mL. $5x10^5$ PBMC/ well were incubated in 96-well tissue culture plates (Greiner, Alphen a/d Rijn, The Netherlands) and stimulated with 1 ng/mL lipopolysaccharide (E.Coli 055:B5, Sigma, St Louis, MO.), 1 μg/mL phytoheaemoagglutinin (PHA-P, Sigma) or 1 μg/mL *M. tuberculosis* sonicate (kindly provided by Dr. A.H.J. Kolk, Royal Tropical Institute, Amsterdam, The Netherlands). PBMC were cultured in the presence or absence of 1,10 or 100 μg/mL of pentoxifylline (Hoechst Pharma) and/or thalidomide (Penn Pharmaceuticals, UK). Thalidomide was dissolved in DMSO at a concentration of 25 mg/mL (2.5 % w/v). In preliminary experiments, DMSO was found to potentiate LPS-induced production of cytokines. Therefore, a similar final concentration of DMSO was used in all cultures performed. After 24 hours of incubation (TNFα, IL-1β, IL-1ra, IL-10) or 72 hours of incubation (INFγ), the supernatants were collected and stored at -70^o C until analysis.

Cytokine measurements
In all samples the concentrations of TNFα, IL-1β, and IL-1ra were measured by specific radioimmunoassays (detection level: 40 pg/mL) as described [16]. Concentrations of IFNγ and IL-10 were measured by ELISA (detection level: 4 pg) according to the manufacturer (Pelikine, CLB, Amsterdam, the Netherlands).

Statistics
Comparisons between related culture samples were performed using the Wilcoxon-signed-rank-test (SPSS 6.3 software). For assessment of possible synergism between pentoxifylline and thalidomide, multilevel regression analysis was performed (SAS 6.12, SAS institute, Cary, NC). P-values were two-sided and the level of significance was set at $p < 0.05$.

Results

Cytokine production; separate use of pentoxifylline and thalidomide.
Unstimulated PBMC did not produce significant quantities of cytokines. Likewise, pentoxifylline or thalidomide did not induce cytokine production by unstimulated human PBMC (not shown). After stimulation with 1 ng of LPS, culture supernatants contained high concentrations of TNFα (mean: 2.2 ± 0.5 ng/mL) and IL-1β (mean: 15.0 ± 6.6 ng/mL). Both pentoxifylline and thalidomide inhibited the LPS-induced production of TNFα in a dose-dependent fashion (**Figure 1A**). At 1 μg/mL, thalidomide was more potent than pentoxifylline ($P < 0.05$). Maximal inhibition with thalidomide was already present at 10 μg/mL. With the highest concentration used, maximal inhibition of LPS-induced production of TNFα was 74% for pentoxifylline compared with 31% for thalidomide ($P < 0.05$). Production of IL-1β was also inhibited by pentoxifylline and thalidomide (**Fig.1B**). However, maximal inhibition of IL-1β was 50% for thalidomide compared with 19% for pentoxifylline ($P < 0.05$). Maximal effects were found with 10 μg/mL of both drugs. IL-1ra production was not modulated significantly by either drug (**Figure 1C**).
After stimulation of PBMC with PHA (1μg/mL), high concentrations of IFNγ (mean: 8995 ± 6220 pg/mL) were found in culture supernatants. Both pentoxifylline and thalidomide inhibited the PHA-induced production of IFNγ (**Figure 2A**). Pentoxifylline demonstrated a dose-dependent effect with maximal inhibition of 84%. Similar to its effect on LPS-induced production of TNFα, thalidomide demonstrated its maximal inhibitory effect at 10 μg/mL, and was less potent than pentoxifylline at 100 μg/mL ($P < 0.01$). In addition to IFNγ, PHA induced production of IL-10 by PBMC (mean: 912 ± 195 pg/mL). Only pentoxifylline had a significant, although modest effect on the production of IL-10 (**Figure 2B**). With 1 and 10 μg/mL of pentoxifylline, 17% inhibition of IL-10 production was found, while at higher concentrations individuals showed more variable responses.

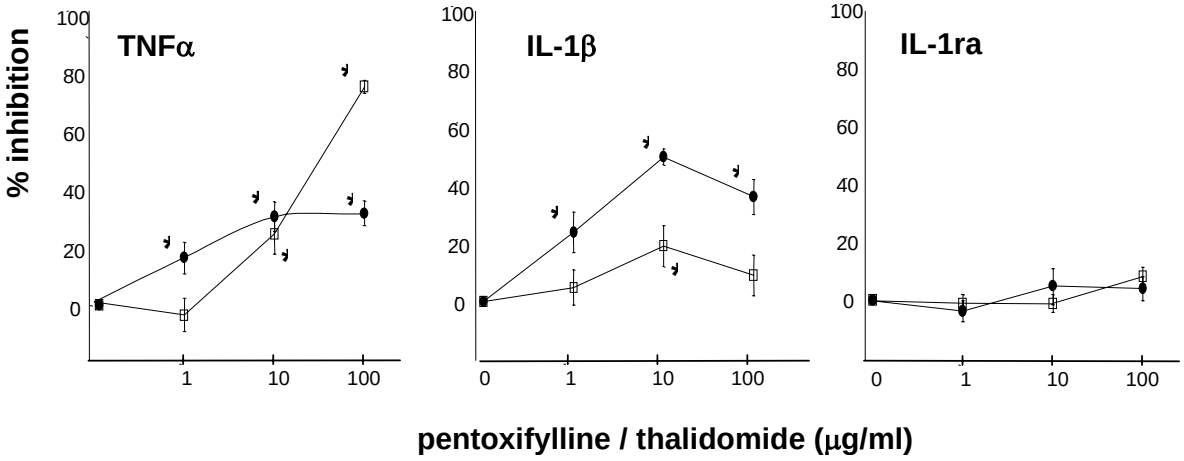

Figure 1.

The effect of thalidomide (closed circles) and pentoxifylline (open squares) on LPS-induced TNFα, IL-1β and IL-1ra production by human PBMC. * $p < 0.05$ vs. baseline.

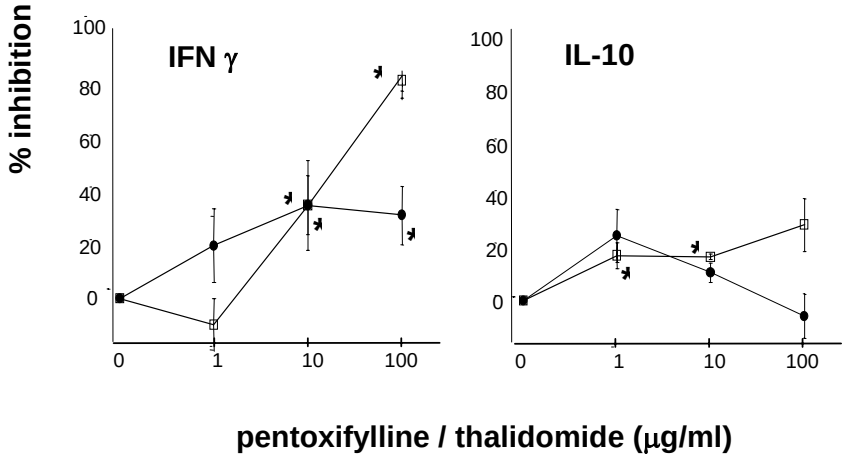

Figure 2.

The effect of thalidomide (closed circles) and pentoxifylline (open squares) on PHA-induced IFNγ and IL-10 production by human PBMC. * $p < 0.05$ vs. baseline.

Modulation of *M. tuberculosis* (MTB) induced cytokine production was also investigated. In these experiments, MTB led to the production of TNFα (2990 ± 1660 pg/mL) and IL-1β (8740 ± 2030 pg/mL), as well as to a variable production of IFNγ (median 8350 pg/mL; range 310 – 29000), and modest production of IL-10 (mean 43 ± 18 pg/mL). Similar to their effect on LPS-induced cytokine production, pentoxifylline and thalidomide inhibited MTB-induced production of TNFα (**Figure 3A**), and IL-1β (data not shown). Thalidomide showed a trend towards a more potent inhibition when used after stimulation with MTB than after stimulation with LPS. However, this difference was not significant. Production of IFNγ after antigen-specific stimulation with MTB was also inhibited by pentoxifylline and thalidomide (**Figure 3B**). Again, the inhibitory effect of thalidomide appeared to be more pronounced after stimulation with MTB than after stimulation with PHA (76% vs. 35% inhibition; $P < 0.05$). With regard to the production of IL-10, we found small and variable effects of pentoxifylline and thalidomide (**Figure 3C**). Pentoxifylline had no significant inibitory effect, while thalidomide at the highest concentration significantly inhibited production of IL-10 (37 ± 16 %).

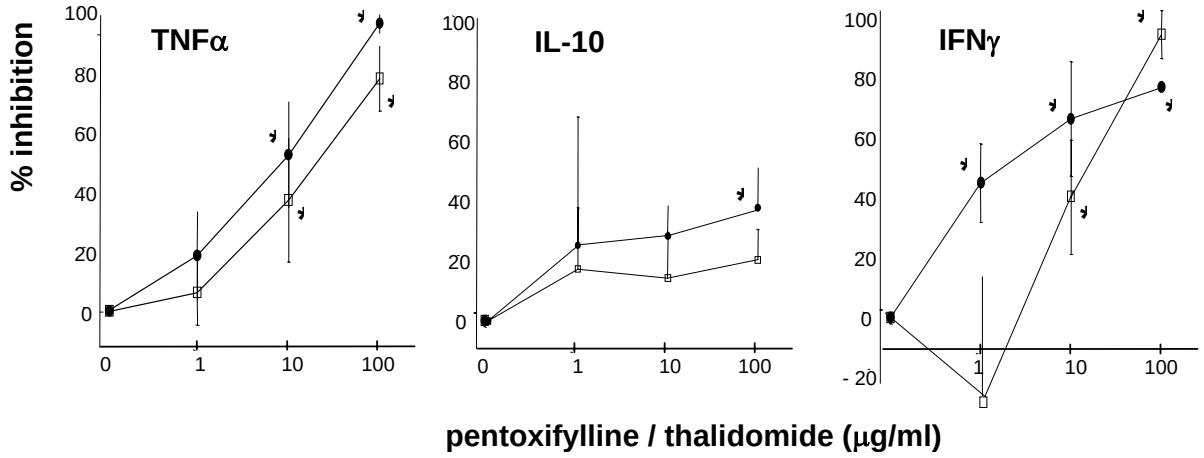

Figure 3.

The effect of thalidomide (closed circles) and pentoxifylline (open squares) on *MTB* sonicate-induced TNFα, IFNγ and IL-10 production by human PBMC. * $P < 0.05$ vs. baseline.

cytokine production; combined use of pentoxifylline and thalidomide.
In the same experiments, pentoxifylline and thalidomide were used together to evaluate possible synergism of inhibition of cytokine response. Pentoxifylline and thalidomide had additive effects on LPS-induced production of TNFα (**Fig. 4A**). No synergistic, as opposed to mere additive effects, exist between the two drugs for the inhibition of TNFα, as demonstrated by multilevel regression analysis ($P > 0.05$). The moderate inhibitory effect of pentoxifylline on the production of IL-1β did not significantly add to the effect of thalidomide alone (not shown). No significant inhibition of the production of IL-1ra was found with combined use of pentoxifylline and thalidomide, even at the highest concentrations. Inhibition of PHA-induced production of IFNγ was more pronounced with the combination of pentoxifylline and thalidomide (**Figure 4B**). However, this inhibition was additive and not synergistic ($P > 0.05$). IL-10 was not inhibited by the combination of pentoxifylline and thalidomide.

When PBMC were stimulated with *M. tuberculosis* sonicate, the combination of pentoxifylline and thalidomide had additive inhibitory effects on the production of TNFα and IFNγ, similar to the additive effect of these drugs after stimulation with LPS or PHA (not shown). Production of antiinflammatory cytokines was not inhibited by the combination of pentoxifylline and thalidomide.

Discussion

The results presented in this study show that after stimulation of PBMC with LPS, both pentoxifylline and thalidomide inhibited the production of TNFα and IL-1β. Pentoxifylline more strongly inhibited TNFα, while thalidomide more strongly inhibited IL-1β. After stimulation with PHA, both agents diminished secretion of IFNγ. No significant modulation of antiinflammatory cytokines IL-10 and IL-1ra was found. When used together, pentoxifylline and thalidomide had an additive inhibitory effect on the release of TNFα and IFNγ. Similar results were achieved when disease-specific stimulation with *M. tuberculosis* sonicate was used.

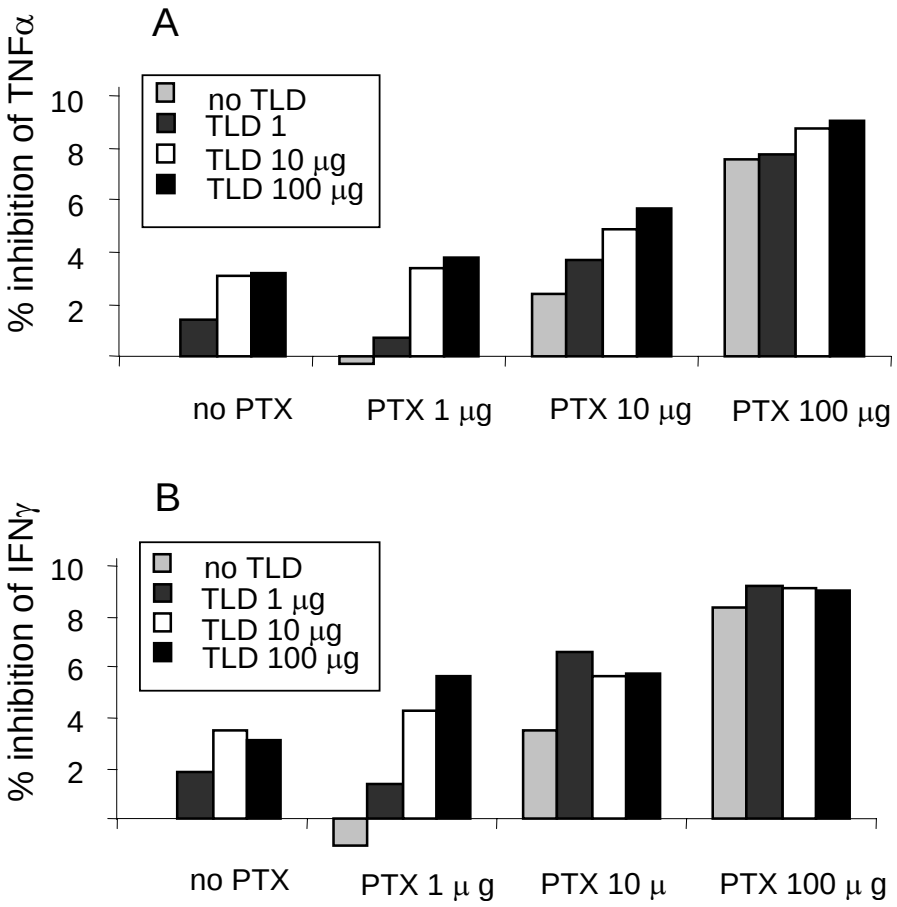

Figure 4.

The combined effect of thalidomide (TLD) and pentoxifylline (PTX) on the LPS-induced production of TNFα (A), and on the PHA-induced production of IFNγ (B).

Using either pentoxifylline or thalidomide, the ex-vivo production of proinflammatory cytokines has been examined under various conditions. The results of the present study demonstrate inhibition of TNFα and of IL-1β by pentoxifylline after stimulation with LPS. This is in line with other studies showing that pentoxifylline downregulates not only TNFα, but also IL-1β [12,14,17], and IL-12 [14]. In this study, as reported before [9,18], thalidomide inhibited production of TNFα, irrespective of the stimulus used. Thalidomide was also found to inhibit IL-1β, which is in accordance with a study demonstrating that potent thalidomide analogues downregulated IL-1β in PBMC [19]. However, according to another report, in which lymphocytes were removed, thalidomide did not affect IL-1β [18]. This suggests that lymphocytes are required for the inhibitory effect of thalidomide on IL-1β.

Both pentoxifylline and thalidomide have been used in mycobacterial infections. In tuberculosis, excess production of TNFα and other proinflammatory cytokines is thought to be involved in detrimental tissue necrosis, and to be responsible for the weight loss, fever and other constitional symptoms. Indeed, thalidomide increased the body weight of patients with active tuberculosis [7], and pentoxifylline moderately ameliorated anemia and general performance in HIV-positive patients with tuberculosis [8]. During treatment of leprosy, erythema nodosum leprosum, a toxic syndrome with systemic manifestations and painful nodules related to former leprosy lesions, can be treated with thalidomide [3] or pentoxifylline [6].

Because of its application in mycobacterial infections, we investigated the modulation of *M.tuberculosis* (MTB) induced cytokine production by thalidomide and pentoxifylline. After specific stimulation of PBMC with MTB, there is a predominant production of proinflammatory cytokines, which was, similar to LPS-mediated cytokine production, inhibited by pentoxifylline and thalidomide. Interestingly, the effect of thalidomide appeared stronger after stimulation with MTB. These effects during mycobacterial stimulation are in accordance with earlier investigations of either pentoxifylline [6,8] or thalidomide [7,18].

Traditionally, immunomodulation by pentoxifylline and thalidomide has been attributed to effects on monocytes. Both drugs may also act by other mechanisms, one of which may be the modulation of T-cell response. Our experiments indicate that the production of IFNγ is inhibited by pentoxifylline and thalidomide, both after unspecific stimulation with PHA, and antigen-specific stimulation with mycobacterial antigens. This is in agreement with earlier reports of in-vitro inhibition of IFNγ by pentoxifylline [17,20] and thalidomide [21]. In contrast, other investigators have found stimulatory effects of thalidomide on IFNγ production when purified T-cell populations

were used [19,22]. Thalidomide has inhibitory effects on monocytes which were absent in these experiments. Pentoxifylline and thalidomide may also affect the production of antiinflammatory cytokines. In our experiments, neither drug modulated the in-vitro production of the natural antagonist of IL-1β, IL-1ra, which has not been described previously. Pentoxifylline slightly inhibited IL-10, while thalidomide had no significant effect. In contrast to our findings, a recent publication reports the upregulation of IL-10 by thalidomide and thalidomide-analogues [19]. However, LPS and not PHA was used as a stimulus in that report, suggesting that the effect on monocytes and lymphocytes may be different. With regard to pentoxifylline, equivocal results have been reported about possible in-vitro modulation of IL-10 [12-14]. In whole blood, LPS- induced production of IL-10 was increased with low concentrations of pentoxifylline, and inhibited with higher concentrations [12]. In another report, modulatory effects of pentoxifylline were related to the stimulus used; LPS-induced production was inhibited, while *S. pneumoniae*-induced IL-10 production was potentiated with pentoxifylline [17]. Clearly, no uniform conclusion can be drawn about the modulation of IL-10 by pentoxifylline and thalidomide.

Since pentoxifylline and thalidomide exert their effects through different mechanisms, the combined use of these substances might be more potent. In vitro, based on experiments using thalidomide with a single dose of pentoxifylline, a synergistic effect was reported [9]. From our experiments with combinations of pentoxifylline and thalidomide in a range of concentrations, it can be concluded that pentoxifylline and thalidomide have additive effects on cytokine production, but that no synergism exists. Combined lower doses of each drug achieving a similar effect may perhaps be safer in clinical practice.

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Chapter 6

Decreased plasma leptin concentrations in tuberculosis patients are associated with wasting and inflammation.

Reinout van Crevel[1], Elvina Karyadi[2], Mihai Netea[1] , Hans Verhoef [3] , Ronald H.H. Nelwan [4], Clive West [3,5], Jos W.M. van der Meer[1].

[1] Dept. of Internal Medicine, University Medical Center Nijmegen, The Netherlands. [2] SEAMEO-TROPMED, Regional Center for Community Nutrition, University of Indonesia, Jakarta, Indonesia. [3] Divisions of Human Nutrition and Epidemiology, Wageningen University, The Netherlands. [4] Working Group on Infectious Diseases, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia. [5] Dept. of Gastroenterology, University Medical Center Nijmegen, The Netherlands.

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Abstract

Tuberculosis patients often suffer from severe weight loss, which is considered to be immunosuppressive and a major determinant of severity and outcome of disease. Because leptin is involved in weight regulation and cellular immunity, its possible role in tuberculosis-associated wasting was investigated. In an urban clinic in Indonesia, plasma leptin concentrations, indicators of adipocyte mass, appetite, C-reactive protein (CRP), tuberculin reactivity and cytokine response were measured in tuberculosis patients and healthy controls. Plasma leptin concentrations were lower in patients than in controls (615 vs. 2550 ng/L; $P<0.001$). Multivariate regression analysis showed that body fat mass and inflammation were two independent factors determining plasma leptin concentrations; there was a positive correlation between fat and leptin, whereas, unexpectedly, leptin was inversely associated with CRP and TNFα-production. Concentrations of both CRP and leptin were independently associated with loss of appetite. Our results do not support the concept that weight loss in tuberculosis is caused by enhanced production of leptin. Rather, loss of body fat leads to low plasma leptin concentrations, and prolonged inflammation may further suppress leptin production. Because leptin is important for cell-mediated immunity, low leptin production during active tuberculosis may contribute to increased disease severity, especially in cachectic patients.

Introduction

Wasting has long been recognized as a prominent feature of tuberculosis, and is probably one of the determinants of the disease severity and outcome [1]. However, uncertainty surrounds cause as well as effect of a poor nutritional status in tuberculosis patients. The cause, or pathogenesis of tuberculosis-associated wasting is incompletely understood, although it is likely that inflammatory mediators such as tumor necrosis factor-α (TNFα) do play a role [2]. Similarly, a poor nutritional status is known to suppress cellular immunity, which is essential against M. tuberculosis, but the precise mechanism remains uncertain [3,4].

Leptin, the 16-kDa product of the ob-gene, may be involved in this cross-regulation between nutritional status and the immune response in tuberculosis. Leptin is produced by adipocytes, and binds to specific receptors in the hypothalamus, from where it suppresses appetite [5]. Concentrations of circulating leptin are proportional to fat mass [6], are reduced in starvation [7,8], and are increased by inflammatory mediators [9]. Administration of leptin to leptin-deficient ob/ob mice reduces food intake and increases energy expenditure [10]. Experimental evidence has shown a number of other possible functions of leptin, including immune regulation. Leptin stimulates the proinflammatory response [11] and promotes proliferation, differentiation and activation of hematopoietic cells [12]. In mice, the reduction of leptin concentrations induced by starvation enhances sensitivity to endotoxic shock [13]. Falling leptin concentrations also appear to be responsible for reduced T-cell function during starvation [14].

On the basis of the above, plasma leptin concentrations in tuberculosis may be the result of two antagonistic mechanisms. Whereas tuberculosis-associated loss of body fat mass may lead to reduced production of leptin [15], the host inflammatory response may increase leptin production [9]. If, as an overall result, plasma leptin concentrations are increased in tuberculosis patients, then this might theoretically suppress appetite and food intake and be one of the mechanisms underlying weight loss. However, if plasma leptin concentrations are decreased in tuberculosis, then this might suppress cellular immunity and aggravate disease outcome. Therefore, the aim of this study was to measure plasma leptin concentrations in tuberculosis patients, and to explore determinants of leptin such as nutritional status and the inflammatory response. The study was conducted in Indonesia, where malnutrition is highly prevalent among tuberculosis patients [3].

Methods

Subjects

In an outpatient tuberculosis clinic in Jakarta, Indonesia, 60 consecutively selected patients with pulmonary tuberculosis were evaluated before and after 2 months of standard antituberculous treatment with isoniazide, rifampicin, pyrazinamide and ethambutol. Diagnosis was based on clinical presentation and radiology, and confirmed by sputum microscopy and culture for M. tuberculosis. In a subgroup of 20 (untreated) patients, tuberculin reactivity was measured. Thirty healthy individuals resident in the same neighborhood as the patients were selected for comparison. These controls had no history or signs of active pulmonary tuberculosis, and had no abnormalities on chest X-ray examination. Informed consent was obtained from all patients and control subjects, and the study was approved by the ethical committee of the Faculty of Medicine, University of Indonesia, Jakarta.

Anthropometric measurements

Patients and control subjects were weighed barefoot with minimum clothing using an electronic weighing scale (SECA-770). Body weight was recorded to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm using a microtoise. Total body fat was estimated from the average of two duplicate measurements of skinfold thickness at four sites (biceps, triceps, subscapular and suprailiac region) [16]. Food intake was estimated from two 24-h recalls using World Food vs 2.0 (University of California, Berkeley, CA).

Laboratory methods

Plasma leptin concentrations were measured by capture ELISA according to guidelines of the manufacturer (Quantikine DLP00, R&D, Minneapolis, MN). Plasma C-reactive protein concentrations (CRP) were measured by standard turbidimetry. In a subgroup of 20 patients, ex-vivo production of cytokines was assessed in whole blood as previously described [17]. Briefly, whole blood was incubated in closed vacutainer tubes at 37 °C without stimulation or with lipopolysaccharide (LPS; final concentration 10 μg/L), phytohemoagglutinin (PHA; 10 mg/L) or PPD (10 mg/L). Supernatants were harvested after incubation for 6 h (LPS and PHA) or 24 h (PPD). Concentrations of TNFα, interleukin-1β (IL-1β) and interleukin-1 receptor antagonist (IL-1Ra) were measured by specific radio-immunoassay [18], and concentrations of IFNγ and interleukin-6 (IL-6) were measured by ELISA (Pelikine, CLB, Amsterdam, The Netherlands). All relevant comparisons were made within single assays. The intraassay CV was 3% for leptin, 6% for IFNγ, and < 10% for TNFα, IL-1β and IL-1ra. Day to day variation of whole blood ex-vivo cytokine production in humans is 12% for TNFα, 23% for IL-1β, 5% for IL-1ra and 47% for IFNγ [17].

Statistical analyses

Data were analyzed using SPSS version 7.5.2 for Windows (SPSS Inc., Chicago, IL). Patients and controls were compared regarding their plasma leptin concentrations, body weight, body mass index (calculated as weight/height2, kg/m2), body fat mass, sex and age, using Student's t-test or Mann-Whitney test as appropriate. The relationship between plasma leptin concentrations and body fat mass, and between plasma concentrations of leptin and CRP was analyzed by univariate regression. Multivariate regression models were used to assess if data were consistent with our hypothesis that a tuberculosis-associated change in plasma leptin concentration is mediated through changes in body fat mass and CRP. Hence, log (plasma leptin concentration) was modeled with body fat mass and CRP as main terms. A term for tuberculosis was retained to account for possible mechanisms through which it might influence plasma leptin concentration independently from body fat and CRP. The geometric mean changes in plasma leptin concentration, food intake, and the mean change in body weight, body fat mass and CRP after 2 mo of antituberculous treatment were evaluated by one-sample t-tests. Multivariate logistic regression analysis was used to evaluate plasma leptin concentrations and inflammation as determinants of reported appetite ('normal' or 'below normal') in tuberculosis patients at baseline. CRP was used as a marker of the inflammatory response, which was considered as a possible confounder in the relation of plasma leptin concentration with appetite.

Results

Plasma leptin concentrations are lower in tuberculosis patients than in controls

The subjects included in this study were mostly young adults and more often male (**Table 1**). Patients with tuberculosis presented with a 2- to 6-month history of respiratory symptoms (100%), fever (60%), night sweats (68%), fatigue (83%) and weight loss (80%). None of the investigated patients was HIV-positive. Reported weight loss ranged from 0 - 25 kg (median, 5 kg). Patients with untreated tuberculosis had substantially lower weight, BMI and body fat mass than controls (**Table 1**). BMI in patients was reduced by 16% compared with controls, and body fat mass percentage (%) by 45%. Patients had a geometric mean (95% CI) plasma leptin concentration of 617 (469-810) ng/L, compared with 2539 (1548-4168) ng/L in healthy control subjects ($P < 0.001$; **Figure 1a**). This difference corresponded with a 76% reduction in patients. Patients were somewhat older than controls, but no association was found between age and plasma leptin concentration among either controls or patients. Women had substantially higher plasma leptin concentrations than men (geometric mean 1938 ng/L compared with 575 ng/L; ratio 3.4, 95% CI 2.0-5.6), but the sex ratio was similar among patients and controls (**Table 1**). Both in male and female subjects, plasma leptin concentrations were significantly reduced in tuberculosis patients (**Figure 1b**).

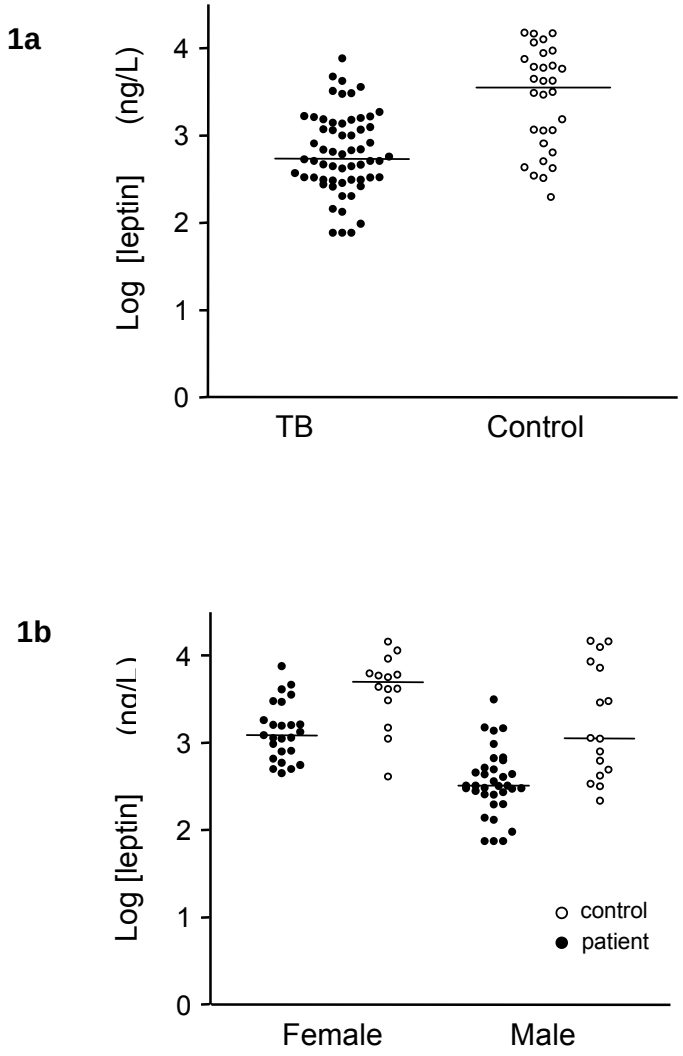

Figure 1.

Plasma leptin concentrations in tuberculosis patients and healthy controls

Log-transformed plasma leptin concentrations of 60 tuberculosis patients (black dots) and 30 healthy controls (open dots) are depicted in figure 1a. Geometric mean plasma leptin concentrations are significantly lower in patients versus control subjects (P<0.001 according to Student's T-test). Figure 1b demonstrates the log-transformed plasma leptin concentrations according to sex. Plasma leptin concentrations are significantly higher in females (P<0.001), but both in males and females, plasma leptin is significanlty reduced in tuberculosis patients.

Table 1.
Nutritional status in patients with untreated tuberculosis and healthy control subjects

	Patients (n=60)		Controls (n=30)		Difference	P
Male / female, % / %	58 / 42		53 / 47			
Age *, y	30.0	[23.3-40.0]	23.0	[19.0-39.3]	7.0	
Weight §, kg	42.2	(40.4-44.1)	49.6	(47.2-52.0)	7.4	<0.001 ‡
Body mass index †, kg m^{-2}	16.8	(16.2-17.4)	20.0	(19.0-21.0)	3.2	<0.001 ‡
Body fat mass *, %	11.8	[7.9-19.1]	21.4	[12.9-26.8]	9.6	0.001 ¶
Body fat mass *, kg	4.6	[3.3-8.4]	9.0	[6.4-14.7]	4.4	<0.001 ¶

* Median, † geometric or § arithmetic mean; *P*-values assessed by ‡ Student's t-test or ¶ Mann-Whitney U-test.
Values between parentheses indicate (95% confidence intervals) or [interquartile range].
Data were available for all tuberculosis patients except for body mass index (n=58) and body fat mass (n=59).

Table 2.
Independent associations of various variables with plasma leptin concentration

Model	Variable	Effect estimates *		95% $CI_{(10^{\beta})}$	**P**
		Regression coefficient, β	Factor (10^{β})		
1	Tuberculosis	-0.628	0.24	0.14-0.39	<0.001
2	Tuberculosis	-0.190	0.65	0.41-1.02	0.06
	10% increase in body fat mass, %	0.432	2.7	2.1-3.4	<0.001
	100 mg/L increase in plasma C-reactive protein concentration	-0.287	0.52	0.34-0.79	0.003

* Effect estimates were first obtained for the regression coefficient (β indicating changes in log (plasma leptin concentration, ng/L); the effect estimates (10^{β}) are proportional instead of additive. Thus, factors indicate the proportional change in plasma leptin concentration adjusted for other variables in the model.
The estimated intercepts in models 1 (univariate) and 2 (multivariate) were 3.405 and 2.540, corresponding to plasma leptin concentrations of 2540 ng/L and 346 ng/L, respectively.

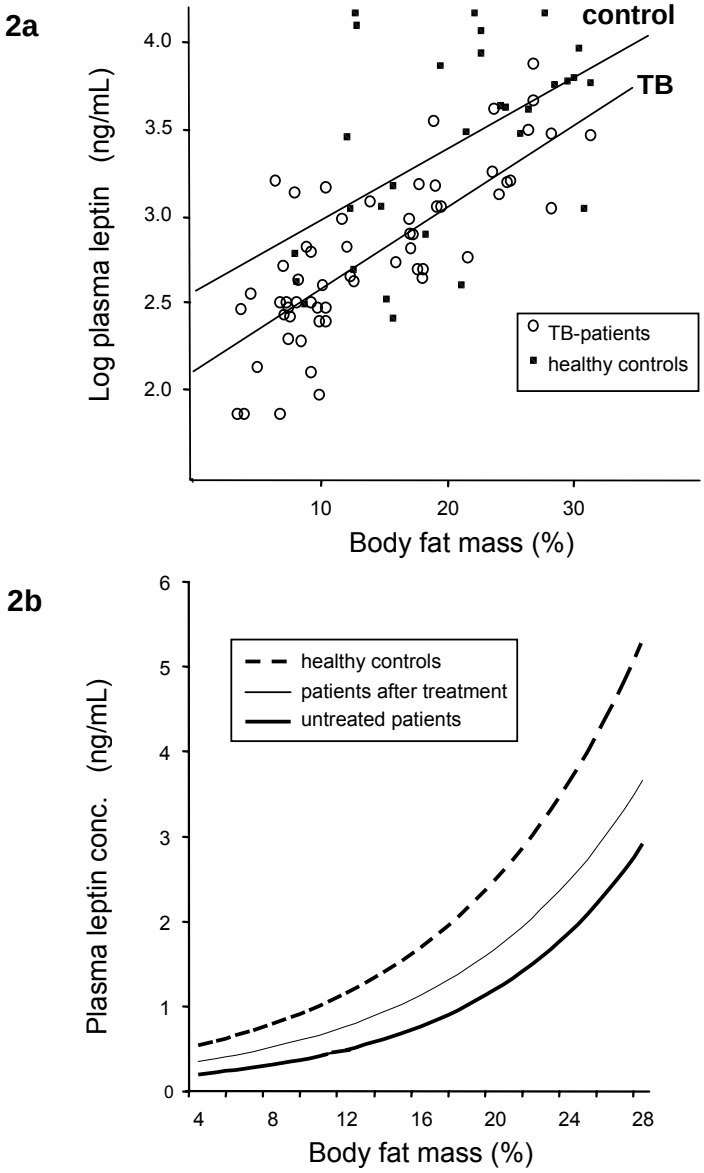

Figure 2.

Plasma leptin concentration and body fat mass in tuberculosis patients and healthy controls.
Fig 2a shows the log transformed plasma leptin concentrations and percent body fat in tuberculosis patients (open circles) and healthy controls (closed squares). The lines represent linear regression analysis for patients ($r = 0.78$; $P<0.001$) and controls ($r = 0.53$; $P=0.002$).
Fig 2b shows the estimates ([Y] = 10 2.1078 + 0.0484 [X] for tuberculosis patients; [Y] = 102.5669 + 0.0414 [X] for healthy controls) based on a multivariate regression model with terms for tuberculosis, body fat mass and their product term. They indicate that tuberculosis affects plasma leptin concentrations directly, and through loss of body fat mass. The thin curve ([Y] = 102.3750 + 0.0424 [X]) is based on univariate regression to model plasma leptin concentration as a function of body fat mass in tuberculosis patients who have completed 2 mo of treatment (n=38). This curve indicates that plasma leptin concentrations show a partial recovery during treatment, which cannot be explained by an increase in fat mass.

Body fat mass and inflammation are associated with plasma leptin concentrations.

Univariate analysis showed that plasma leptin concentration increased proportionally with body fat mass (**Figure 2a**). For every 10 units increment in fat percentage, plasma leptin concentration increased 3.1-fold (95% CI: 2.4-3.9) and 2.6-fold (95% CI: 1.4-4.7) in patients and control subjects, respectively. Median CRP was 52 mg/L (interquartile range: 19-95 mg/L) in tuberculosis patients and 2 mg/L (1-3 mg/L) in controls (P<0.001). Among patients, plasma leptin concentrations were reduced by 33% (95% CI: 15-46%) for every 50 mg/L increment in CRP. In control subjects, there was insufficient variation in CRP to assess its relation with plasma leptin concentration.

Figure 2b shows the results of a multivariate regression model to assess the role of body fat mass and possible other mechanisms whereby tuberculosis may affect plasma leptin concentration. This figure shows that patients with untreated tuberculosis have lower plasma leptin concentrations than expected for body fat mass. **Table 2** gives a comparison of two regression models to assess the role of body fat mass, CRP and possible other mechanisms whereby tuberculosis may affect plasma leptin concentrations. Multivariate regression analysis showed no evidence for effect modification or confounding by age and sex in these associations. Univariate regression (Model 1) gave a crude assessment of the association between tuberculosis and plasma leptin concentration. Multivariate regression, with addition of CRP and body fat mass (Model 2), reduced this association (from 76% to 35%) and shows that plasma leptin concentration was positively associated with body fat and inversely associated with CRP. The estimated proportion of variability in plasma leptin concentration explained (multiple R2) in Model 2 was 0.64. Taken together, these findings are consistent with our hypothesis that tuberculosis-associated reductions in plasma leptin concentrations are mediated independently through a decreased percentage of body fat, inflammation and possibly other, unidentified mechanisms.

Plasma leptin concentrations increase during antituberculous treatment

The effect of antituberculous treatment on plasma leptin concentrations, appetite, nutritional status and acute phase proteins was evaluated in 38 patients for whom blood test results were available after 2 mo of treatment. These patients reported a clear improvement of symptoms within 1-3 weeks after start of treatment. Plasma leptin concentrations were substantially higher following treatment (geometric mean difference: 409 ng/L; 95% CI: 219-676 ng/L; P<0.001), corresponding to an increase of 64% relative to baseline (Figure 2b). There was no evidence that selection bias caused by missing data substantially affected the estimated effect of treatment on the change in plasma leptin concentration (not shown). Loss of appetite was reported by 27 patients (71%) before treatment, and by none following treatment. After 2 months of treatment, patients had a higher energy intake (mean

difference: 375 kJ), body weight (mean difference: 1.6 kg; 95% CI: 0.9-2.3 kg, P<0.001), and body fat mass (%) (mean difference: 1.6%, 95% CI: 0.6-2.5 %, P=0.003), and lower CRP (mean difference: 50 mg/L; 95% CI: 26-74 mg/L; P=0.001).

Plasma leptin and cytokine response

The relationship between plasma leptin concentrations and the ex-vivo production of proinflammatory cytokines was investigated in 19 tuberculosis patients for whom data were available. Linear regression analysis showed an inverse correlation between spontaneous ex-vivo production of TNFα and plasma leptin concentrations before treatment. Plasma leptin concentrations reduced by 42% (95% CI: 12%-62%) for every 0.1 ng/mL increment in plasma TNFα concentration. LPS-mediated production of TNFα, as well as the production of IL-1β, IL-1Ra, and IL-6 were not significantly associated with plasma leptin concentrations before treatment. Following treatment, there was a substantial decrease of plasma concentrations of IL-6, and ex-vivo production of IL-1β, IL-6 and TNFα (not shown). However, we could not directly demonstrate a significant association between changes in leptin concentrations and changes in cytokine production.

To investigate the relationship between plasma leptin concentrations and T-cell immunity, tuberculin skin tests and production of IFNγ were evaluated in untreated patients. The size of skin reactions to PPD showed a positive but statistically non-significant correlation with plasma leptin concentrations (P=0.30). A similar result was found for plasma leptin concentrations and PPD-induced ex-vivo production of IFNγ (P=0.38). Ex-vivo production of IFNγ increased following treatment (mean difference: 188 pg/mL; 95% CI: -7 to 539 ng/L). However, no significant relationship could be shown between change in plasma leptin concentrations and IFNγ production (P =0.39).

Determinants of appetite

Both inflammation and plasma leptin concentration were associated with loss of appetite in tuberculosis patients. Every 50 mg/L-increment in CRP was associated with a 1.4-fold increase (95% CI: 0.8-2.6) in the odds of reporting loss of appetite. When adjusted for CRP, every 1000 ng/L increment in plasma leptin concentration was associated with a 1.7-fold increase (95% CI: 0.7-4.3) in the odds of reporting loss of appetite.

Discussion

Tuberculosis often leads to severe weight loss (wasting), probably through the production of inflammatory mediators [2]. Wasting, in turn, affects the inflammatory response, suppresses cellular immunity and aggravates the outcome of tuberculosis [19]. In these complex relations between tuberculosis, nutritional status and the host immune response, leptin is a possible mediator. In this study, plasma leptin concentrations were significantly suppressed in tuberculosis patients in Indonesia. Body fat mass was strongly correlated with plasma leptin concentrations, both in patients and controls. Unexpectedly, in tuberculosis patients, plasma CRP and in-vitro production of TNFα showed an inverse correlation with plasma leptin concentrations. Results of multivariate regression analysis support the hypothesis that tuberculosis-associated reductions of plasma leptin were mediated independently by weight loss and inflammation. Although previous data have shown that leptin stimulates cell-mediated immunity, we were unable to demonstrate a statistically significant correlation of plasma leptin concentrations with tuberculin reactivity or IFNγ production.

To our knowledge, there is one previous study on plasma leptin concentrations in tuberculosis patients [20]. In that report, leptin concentrations, as determined by radio-immunoassay, were much higher than in ours. The (Turkish) patients in that report had a much higher body mass index, but it seems surprising to us that after treatment they had three-fold higher leptin concentrations than control subjects. Also, the control subjects had increased plasma TNFα values, an unexpected finding in healthy individuals.

Loss of body fat mass could not entirely explain the observed low plasma leptin concentrations in tuberculosis patients in our study. Body fat mass is the most important determinant of plasma leptin concentrations, but starvation, hormones (including insulin and cortisol), as well as inflammatory mediators are able to modulate leptin production [21]. Animal studies have shown that LPS, TNFα and IL-1β raise leptin concentrations in serum and leptin mRNA in adipose tissue (7). Similarly, in cancer patients, recombinant TNFα (22) and IL-1β (23) increased plasma leptin. In sepsis patients, leptin levels were found to be elevated [23-25]. To our surprise, in our study in tuberculosis, CRP and TNFα production were inversely correlated with plasma leptin concentrations. Attenuation of the acute phase response and proinflammatory cytokine production during antituberculous treatment was accompanied by an impressive increase of plasma leptin concentrations. Of course, the acute inflammatory response in the animal and patient studies described above is different from the more chronic response in tuberculosis patients. The pattern of plasma leptin concentrations in weeks or months prior to diagnosis remains unknown, but one may hypothesize that the prolonged inflammatory response in tuberculosis downregulates or “exhausts” leptin production.

In this study, multivariate analysis indicated that plasma leptin concentrations were associated with loss of appetite in tuberculosis. However, plasma leptin concentrations were substantially higher in control subjects (without anorexia), and patients regained appetite during treatment, in spite of a substantial increase in plasma leptin concentrations. Therefore, anorexia in tuberculosis seems to be determined to a much larger degree by inflammatory mediators (e.g. pro-inflammatory cytokines) than by leptin. Leptin signals the brain to decrease food intake, but sofar no evidence has been found that anorexia in AIDS [26,27] and other inflammatory disorders is caused by increased leptin levels [28,29]. In fact, it may be the other way around: both in laboratory animals [30] and human subjects [8], fasting induces falling leptin levels which evoke a number of adaptive responses, including suppression of metabolic rate [7]. Similarly, in tuberculosis, decreased energy-intake may reduce leptin production. We did not measure energy-intake, but it is likely to be lower among tuberculosis patients than healthy controls.

Suppressed production of leptin may be detrimental for host defense against infections. In septic shock, mortality was found to be associated with decreased plasma leptin levels [22]. In an animal model, the absence [13], or starvation-induced down-regulation of leptin increased susceptibility to endotoxic shock, and leptin partially reversed this effect [13]. In addition, leptin reversed starvation-induced T-cell suppression- [14]. Host defense against tuberculosis depends on cell-mediated immunity, with a crucial role for Th1-type cytokines, primarily IFNγ [31]. Therefore, it may be hypothesized that decreased leptin production during active tuberculosis contributes to T-cell unresponsiveness. Indeed, in our patient group both plasma leptin and ex-vivo IFNγ production were low and increased upon successful antituberculous treatment. We did not find a significant correlation between these two variables, which might be due either to the limited number of patients analyzed for cytokine production, or to substantial intra- and inter-individual variation of ex-vivo cytokine production [17]. We were also unable to show a statistical association between leptin and tuberculin reactivity, but skin testing, which was only done before treatment, is a rather crude measurement.

Based on our data and results from previous studies we hypothesize that in untreated tuberculosis, loss of body fat, reduced energy-intake and the host immune response reduce leptin production (**Figure 3**). As leptin is important for cell-mediated immunity, suppressed leptin concentrations may contribute to a worse outcome of tuberculosis, especially in cachectic patients. In theory, administration of leptin might benefit tuberculosis patients, but this is not feasible in a country like Indonesia. Supplementation of micronutrients such as vitamin E [32] or zinc, which are known to increase leptin production [33], might be a cost-effective alternative. Of interest, zinc has the additional advantage of stimulating appetite [34].

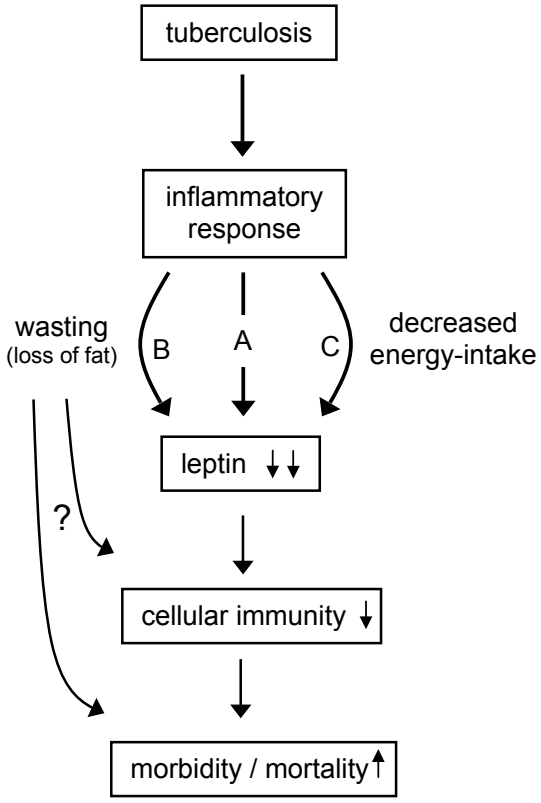

Figure 3.
Hypothesised role of leptin in human tuberculosis.

The inflammatory response in tuberculosis may suppress leptin production directly (A), and through loss of body fat mass (B) and decreased energy intake (C). Suppressed leptin production may contribute to decreased cell-mediated immunity (CMI). In addition, wasting (cachexia) may contribute to a worse disease outcome through other, undefined mechanisms.

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Chapter 7

The impact of nontuberculous mycobacteria on the management of patients with presumed pulmonary tuberculosis.

Reinout van Crevel[1], Wiel C.M. de Lange[2], N.A. Vanderpuye[1], D. van Soolingen[3], J.A.A. Hoogkamp-Korstanje[4], M. van Deuren[1], B.J. Kullberg[1], C. van Herwaarden[2], J.W.M. vander Meer[1].

[1] Dept. of Medicine, [2] Dept. of Pulmonolgy, [4] Dept of Medical Microbiology, University Medical Center Nijmegen, The Netherlands. [3] Reference Laboratory for Mycobacteria, National Institute for Public Health and the Environment, the Netherlands.

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Abstract

The presence of non-tuberculous mycobacteria (NTM) in sputum or bronchial washings may cause diagnostic problems, which affect clinical management. In a retrospective analysis of 135 patients in a Dutch tuberculosis center, patients with NTM isolates were thoroughly investigated. Colonization or contamination by NTM was differentiated from true lung disease. 25 HIV-seronegative and two HIV-seropositive patients with NTM were identified. In only 14 (52%), NTM were a likely cause of disease. In 15 (55%), their presence led to preliminary diagnosis and treatment of tuberculosis. Unnecessary or inappropriate treatment was instituted in 17 (63%) of patients with NTM. Also, in two patients, NTM in sputum led to delay in diagnosing malignant disease. In this series, NTM in sputum or bronchial washings poorly reflected disease, and often led to diagnostic and therapeutic errors. Although it is common knowledge that the presence of NTM should be considered in smear-positive patients, this apparently is a diagnostic pitfall in clinical practice. Reliable DNA-based techniques and better communication between physicians and microbiologists may improve management of suspected mycobacterial infections.

Introduction

Mycobacterial species not belonging to *Mycobacterium tuberculosis* complex and *M. leprae* are designated ‘atypical‘ or non-tuberculous mycobacteria (NTM) [1]. Human transmission of these bacteria, which are ubiquitous in soil and water, is rare [2]. The pathogenicity of non-*M. tuberculosis* complex species varies. *M. avium, M. kansasii* and *M. malmoensae* are considered the most pathogenic, particularly in patients with pre-existent lung disease, past tuberculosis, systemic immunosuppression or the acquired immuno deficiency syndrome (AIDS) [3]. Incidentally, NTM may cause pulmonary disease in immunocompetent individuals [1,4]. In addition, NTM may also colonize the lungs without causing disease [2]. Due to their ubiquitous nature, coincidental isolation or contamination of diagnostic samples may also occur [5-7].

On direct microscopic examination, NTM are acid-fast bacilli (AFB), which cannot be distinguished from *M. tuberculosis.* Therefore, pulmonary tuberculosis may falsely be suspected [6]. When NTM are cultured from pulmonary specimens, non-tuberculous mycobacterial disease still must be differentiated from contamination or colonization. Thus, detection of AFB in pulmonary specimens or the isolation of NTM may pose a diagnostic problem to the clinician who has to decide whether treatment is indicated or not. In recent years this issue has become even more important as NTM are detected in sputum or bronchial washings with increasing frequency.

The present study evaluates the clinical impact of the detection of NTM in pulmonary specimen. To this end we studied in detail the medical records of all 27 patients with NTM in pulmonary specimen in a group of 135 consecutive patients referred to our tuberculosis center with presumed pulmonary tuberculosis.

Patients and methods

Setting

The study was performed in the University Lung Center Dekkerswald of Nijmegen University, one of the two Dutch tuberculosis centers for prolonged in-hospital treatment of tuberculosis patients. Nearly all patients in this center are referred from other hospitals, principally because of diagnostic problems, severe or complicated disease, or treatment problems like drug-resistance, drug-toxicity or non-compliance.

Study design

The medical records of all consecutive 135 patients referred from January 1995 to December 1998 with presumed or confirmed pulmonary tuberculosis were studied retrospectively. From this sample, we further evaluated 90 patients in whom mycobacteria had been identified by direct microscopy or culture of sputum or bronchial washings, either before or after referral to our tuberculosis center. The following characteristics were collected: demographic data; the presence of risk factors for NTM disease (pre-existent lung disease, immunosuppressive medication, HIV-infection or other immunocompromising diseases); signs and symptoms like cough, hemoptysis, weight loss, fever and duration of symptoms; chest X-rays; bacteriological results; treatment; and outcome.

Patients who had NTM isolated from a pulmonary specimen were compared with patients with proven *M. tuberculosis* pulmonary infection. The consequences of finding NTM were evaluated in all patients individually. For the diagnosis of non-tuberculous lung disease, the diagnostic criteria of the American Thoracic Society (ATS) were used [8].

Microbiology

In 76 patients the initial diagnostic procedure and the initiation of therapy had been performed in the referring hospital. In these cases the microbiological results of this hospital were used. The diagnostic procedure in the tuberculosis center comprised of a direct examination for acid-fast bacilli (AFB) in a fluorochrome- and Ziehl-Neelsen stained smear, and of cultures on conventional solid media (Löwenstein and Colestos) and liquid media (BACTEC). In all patients sputum examinations were repeated at weekly intervals. All isolates, either obtained in the referring hospital or the tuberculosis center were sent to the Dutch National Institute of Public Health and Environment (RIVM) in Bilthoven, the Netherlands, for identification, DNA fingerprinting and determination of drug susceptibility [9].

Statistics.

Groups were compared statistically by using a Chi-square test or a non-parametric Mann-Whitney-U test. A P-value < 0.05 was considered significant.

Results

Microbiology

Among the 90 patients evaluated, NTM was isolated from sputum or bronchial washings in 22 and *M. tuberculosis* (MTB) in 63. In four patients, not further included in the comparison between the NTM- and the MTB-group, both NTM and MTB were cultured. The isolated NTM were *M. avium* (n = 11), *M. malmoense* (n = 3), *M. kansasi* (n = 2), *M. terrae* (n = 2), *M. gordonae* (n = 1), and *M. xenopi* (n = 1). Fast growing NTM *(M. peregrinum* or *M. chelonae*) were found in two patients. In five cases species identification was incomplete; culture and PCR for MTB or *M. avium* were negative.

PCR for MTB on sputum or bronchial washings was performed in nine patients with NTM. In agreement with the results from culture and conventional species differentiation, seven were negative for MTB. In two patients, PCR appeared to be false positive for MTB: in one patient MTB was never cultured while all other specimens were positive for *M. avium*, in the other the chest-X-ray abnormalities disappeared after treatment with a short course of amoxicillin-clavulanic acid.

Patient characteristics

Patients with NTM were significantly older (mean age 76 vs. 37 years) than patients with tuberculosis and originated less often from countries with a high prevalence of tuberculosis (13% vs 63%; $P < 0.05$). (**Table 1**). As compared to the patients with pulmonary tuberculosis, NTM-patients sligthly more often suffered from chronic obstructive pulmonary disease (26 *vs.* 11%, n.s.), and were more often on immunosuppressive medication (30 *vs* 5%; $P < 0.05$). 44% of patients with NTM had completed treatment for tuberculosis in the past. However, in more than half (52%) of the NTM-patients not any of the known risk factors for NTM-infection were present. Fever was present in a minority of NTM patients (26% vs. 51%, $P < 0.05$). No significant differences were found with respect to the presence of cough or hemoptysis. Solitary nodular lesions on the chest X-ray were found in four patients (18%) with NTM isolates as compared to none in patients with MTB ($P < 0.05$). However, cavitary disease was observed slightly more frequently in MTB (44%) as compared to patients with NTM (35%) (n.s.). The percentage of patients with AFB on direct examination of pulmonary specimen (the smear-positive rate) was similar for NTM and MTB, but in patients with NTM, AFB were more often found in bronchial washings only (43% vs. 11%; $P < 0.05$).

Table 1

Characteristics of 86 patients with ***M. tuberculosis*** (MTB) or non-tuberculous mycobacteria (NTM) in sputum or bronchial washings[1].

	MTB (n=63)		NTM (n=23)
Average age (years)	37	*	62
male	71 %		74 %
Originating from TB-endemic areas	63 %	*	13 %
HIV-positive	4 %		0
Immunosuppressive medication	5 %	*	30 %
pre-existing lung disease	11 %		26 %
fever (>38.5 ^{o}C)	51 %	*	26 %
Cough	77 %		78 %
Hemoptysis	30 %		22 %
Pulmonary cavities	44 %		35 %
Solitary nodule on chest X-ray	0 %	*	18 %
positive for acid-fast-bacilli	79 %		70 %
- in bronchial washings only	11 %	*	43 %

[1] The 4 patients in whom both *M. tuberculosis* (MTB) and non-tuberculous mycobacteria (NTM) were present, are not included in this Table. * significant difference; $p < .05$.

True infection with NTM versus contamination or colonization

Based on the ATS-criteria [8], the presence of NTM was unlikely to reflect true non-tuberculous lung disease in 11 (48%) of the 23 patients with NTM. Three patients were presumably temporarily colonized with NTM, as they had *M. avium, M. peregrinum* or *M. malmoense* in more than one specimen, but did not fulfill ATS-criteria and failed to show progressive disease during follow-up in the absence of treatment.

Contamination of specimens with NTM seemed most likely in eight patients, all with one single positive specimen. Five of them had been referred by the same hospital within several months. Bronchial washings in these patients contained AFB, for which culture and PCR for MTB and *M. avium* were negative. Contamination with NTM was suspected when AFB were repeatedly detected in the washing machine for bronchoscopes. In three additional patients, only one single positive sputum culture (*M. terrae* (2), *M. xenopi*) was found despite repeated examinations.

In the four cases hwere NTM together with MTB were isolated from pulmonary specimen, NTM reflected true disease in two. Both patients had *M. avium* disease**.** In these two patients, one HIV-seropositive, acid-fast bacteria reappeared and persisted

in sputum during anti-tuberculous treatment. *M. avium* was cultured, only to disappear after ofloxacin and clarithromycin had been added to the treatment regimen. In two other cases with NTM (*M. malmoense* or *M. gordonae*) and MTB isolated from the same patient, contamination of specimens or culture was most likely; NTM were isolated only once.

Patients with true NTM lung disease more often suffered from pre-existing lung disease (36% *vs.* 15%), previous pulmonary tuberculosis (50% *vs.* 38%), hemoptysis (36% *vs.* 15%) and pulmonary cavities (57% *vs.* 22%) than NTM-patients, which did not fulfill criteria for true pulmonary disease. Use of immunosuppressive medication was similar (28% and 30%). *M. avium, M. kansasii* and *M. malmoense* were most frequently found in patients fulfilling criteria for true pulmonary disease (**Table 2**). Microscopic examination of specimens was positive for AFB in a similar percentage of patients, although smear-positive sputum reflected true disease twice as often.

Impact of NTM in pulmonary specimens on diagnosis and treatment.
The presence of NTM often led to diagnostic and therapeutic errors (**Table 2**). In 14 out of 22 patients from whom NTM were cultured, and in all five cases where bronchoscopes were thought to have contaminated diagnostic specimens with NTM, the initial working diagnosis was different from the the final diagnosis, made with all available information. Initially, 15 out of the total of 27 patients with NTM in pulmonary specimens (55%) were falsely considered having pulmonary TB. This group included 6 patients with true non-tuberculous infections and nine patients in whom NTM most likely reflected contamination. All were started on treatment against MTB. Acid-fast bacteria reappeared in sputum from two patients with culture-positive cavitary tuberculosis after two months of anti-tuberculous therapy. These patients were consequently treated for presumed multi-drug-resistant TB until culture demonstrated *M. avium* one, respectively three months later. All together, anti-tuberculous treatment (median: 6 weeks, range: 3 days – 4 months) was unnecessarily instituted in nine patients (33%) and inappropriate drug-regimens (median: 8 weeks; range 1 – 3.5 months) were unintentionally prescribed to eight patients (29%). Four of these patients developed a rise in serum aspartate transaminase (AST) of more than three times the normal upper limit due to the anti-tuberculous therapy.

In two patients, a false positive diagnosis of tuberculosis led to a 6 weeks delay in the diagnosis and treatment of malignant disease. The first patient, who was under immunosuppressive therapy for cryoglobulinemia, presented with infiltrative and cavitary abnormalities on a chest X-ray. *M. avium* was cultured from sputum, but no improvement was seen on antimycobacterial therapy. Finally, a lung biopsy was diagnostic for adenocarcinoma. The second patient, a 65-year old man using prednisone for glomerulonephritis, presented with a solitary cavitary nodule. Bronchial washing yielded AFB and anti-tuberculous treatment was started. Culture

for mycobacteria remained negative and a lung biopsy, performed six weeks after the start of treatment, revealed adenocarcinoma.

Table 2.
Impact of finding non-tuberculous mycobacteria (NTM) in sputum or bronchial washings in 27 patients [1], as divided in NTM lung disease [2], and colonization or contamination of NTM.

	True NTM lung disease	**Contamination or Colonization with NTM**
Species determination	*M. avium (9)* *M. malmoense (2)* *M. kansasii (2)* *M. cheloni*	*M. avium (2)* *M. malmoense* *M. xenopi* *M. peregrinum* *M. terrae (2)* *M. gordonae* *Not specified (5)* [3]
Diagnostic errors:		
suspected pulmonary TB	6 /14	9 /13
suspected MDR-TB	2 /14	
suspected NTM-disease		2 /13
delay of true diagnosis [4]		2 /13
Therapeutic errors:		
unnecessary treatment [5]		9 /13
inappropriate regimen [6]	8 /14	

MDR = multi-drug resistance. [1] including 4 patients, with both MTB and NTM. [2] true lung disease due to NTM was diagnosed according to ATS-criteria [8]. [3] no mycobacteria were cultured; repeated examination confirmed the presence of AFB, due to contamination of bronchoscopes (see text). [4] adenocarcinoma (see text). [5] median: 7 wk. [6] median: 6 wks.

Discussion

In this study diagnostic problems related to the presence of NTM in sputum or bronchial washings from patients with presumed pulmonary tuberculosis were analyzed. In this population with a low prevalence of HIV-infection, NTM, although highly prevalent, poorly reflected true disease, and led to delay of true diagnosis and even referral to a specialized TB hospital. Often, pulmonary tuberculosis or multi-drug resistance were falsely suspected, as a result of which inappropriate or unnecessary anti-tuberculous therapy was prescribed. Apparently, despite the fact that it is common knowledge that the presence of AFB in pulmonary specimen may reflect NTM, this can still be a diagnostic pitfall in clinical practice.

When AFB are present in pulmonary specimens, physicians may have to decide about therapy before mycobacterial culture and species determination has been performed. The risk of untreated active tuberculosis must be weighed against the consequences of an incorrect diagnosis. In our series, demographic and clinical parameters did not prove helpful in differentiating MTB from NTM. Reportedly, the presence of diffuse nodular disease on chest X-ray, found in two patients in this series, and the occurrence of vanishing infiltrates in elderly women, the so-called *"Lady Windermere syndrome"* are characteristic for NTM-disease [1,11]. However, these radiographic abnormalities are not specific. Unlike culture, which may take weeks, microscopic examination of AFB does not differentiate MTB from NTM either. PCR is able to rapidly differentiate MTB from NTM-isolates, and DNA-fingerprinting can identify contamination. However, PCR led to a false-positive diagnosis of pulmonary tuberculosis in two patients in this series, as has been demonstrated in other settings as well [12,13]. Clearly to date, these techniques, as well as their use in clinical practice, may still be improved.

Even when AFB in pulmonary specimen are recognized as NTM, it remains difficult to discriminate true disease from colonization or contamination by NTM [14]. Of all *M. malmoense* isolates in the United States between 1993 and 1995, only 10% reflected true disease [15]. The new and more sensitive laboratory methods may yield even more false-positive cases due to colonization of the patient [1] or contamination of diagnostic specimens [5-7]. In our series, the presence of NTM in hospital tap water contaminating bronchoscopes led to a false positive diagnosis in five patients [16]. The high prevalence of NTM in the present study is in agreement with other reports [14,17], although it should be noted that the current study included a selected patient group that was referred to a tuberculosis center because of diagnostic or therapeutic difficulties.

The presence of NTM in our patient group had a substantial impact on clinical management. False diagnosis of pulmonary TB due to the presence of NTM led to inappropriate or unnecessary treatment in 63% of patients with NTM-isolates. In addition, multi-drug resistance was falsely suspected in two patients, and the presence of NTM in diagnostic specimens delayed the diagnosis of pulmonary malignancies in two patients. Especially when sputum is negative and bronchial washings are positive, the odds of isolating NTM rise. Using the ATS-criteria of NTM lung disease [8], the significance of finding NTM should be thoroughly evaluated before treatment is started. In this respect, careful and repeated bacteriological examination, and good communication between physician and microbiologist, are crucial.

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Chapter 8

Diagnosis and treatment of tuberculosis in Indonesia; an observational study in a tuberculosis control clinic in Jakarta

Reinout van Crevel[1], Halim Danusantoso[2], Bachti Alisjahbana[3], D. van Soolingen[4], J.W.M. vander Meer[1], Ron H,H, Nelwan[5].

[1] Department of Medicine, University Medical Center Nijmegen, The Netherlands. [2] Perkumpulan Pembertasan Tuberkulosa Indonesia (PPTI), Jl Baladewa, Jakarta, Indonesia. [3] Department of Internal Medicine, Padjadjaran University, Bandung, Indonesia. [4] The National Reference Laboratory for Mycobacteriology, National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands. [5] Working Group on Infectious Diseases, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia.

Submitted

Abstract

Indonesia has the third highest tuberculosis caseload worldwide, but little is known about important aspects of this re-emerging epidemic, like quality of diagnosis and treatment, HIV-infection and drug resistance of MTB isolates. In a TB control clinic in Jakarta, we assessed the clinical and microbiological status of a cohort of Indonesian patients. In 121 patients with suspected tuberculosis, microscopy of three large-volume sputum samples was positive in 97 cases (80%). Most patients presented with longstanding and extensive disease. HIV-infection was diagnosed in 1%, while malnutrition (BMI < 17) was found in 50% of cases. Of 146 non-selected patient isolates, 24.7% was resistant to INH and 6.1% to rifampicin. Resistance to at least one first-line drug was present in 25.9% of new cases and 39.5% of retreatment cases. During treatment, side effects were common, but hepatotoxicity was completely absent. 10% of patients defaulted treatment. The death rate was 7%, and was strongly related to malnutrition. This study provides a detailed characterization of tuberculosis patients in an Indonesian clinic. Sputum microscopy has a high diagnostic yield in this endemic setting where most patients present with advanced disease. The drug-resistance patterns found threaten the success of first-line antituberculous treatment and warrant implementation of more widespread susceptibility testing of strains in Indonesia. Because no hepatotoxicity was found, patients' compliance and quality of drug-treatment need further evaluation.

Introduction

In 1998 Indonesia had the third highest tuberculosis caseload in the world [1]. Unfortunately, according to WHO, only 12.2% of new smear-positive cases were detected, and only 54% of smear-positive patients were successfully treated [1]. The impaired economic situation in Indonesia most likely contributes to this unfavorable situation, but other factors may be important as well.

The first step in identification and treatment of tuberculosis patients is detection of acid-fast bacilli in sputum samples. In high prevalence countries, sputum smear positive cases have been given the first priority in directly observed treatment, short-course (DOTS) programs. In recent hospital surveys in Indonesia, sputum was only positive for acid-fast bacilli in 25-50% of patients treated for pulmonary tuberculosis [2-4]. After a diagnosis is made, a full course of antituberculous drugs needs to be taken. DOTS has been shown to improve patients' adherence to treatment and proved very effective in South Sulawesi, an island in the Eastern part of Indonesia [5]. However, potential spread of multidrug resistant tuberculosis poses a serious threat to the success of DOTS. Indonesia is not included in the worldwide drug resistance surveillance [6]. For economic and technical reasons, drug susceptibility testing is usually not performed, but limited investigations suggest that multidrug resistance may be more than 10% [7]. Other factors may hamper the management of tuberculosis in Indonesia. Malnutrition, which is highly prevalent among tuberculosis patients in Jakarta [8], affects the outcome of tuberculosis [9]. HIV-infection, which increases susceptibility for tuberculosis, is on the increase in Indonesia, but little information is available from tuberculosis patients.

From the above it is clear that various diagnostic and therapeutic aspects of tuberculosis in Indonesia need further investigation. We conducted an explorative study in an outpatient tuberculosis clinic in Central Jakarta. Our findings may apply to other low-income urban settings in and outside Indonesia.

Methods

patient inclusion, diagnosis and follow-up

This study was conducted in an outpatient tuberculosis clinic in a densely populated area of Jakarta (PPTI, Jl Baladewa). This clinic provides free anti-TB-treatment and generally attracts patients from low socio-economic class. The majority of patients present themselves spontaneously, but some are referred by health-workers elsewhere, mainly for economic reasons. Therefore, patients presenting to this center may be a selected group with an increased rate and severity of tuberculosis.

From December 1998 through March 1999, based on chest X-ray and history, 121 consecutive patients with suspected tuberculosis were included. Demographic data, symptoms and signs, risk factors for tuberculosis, and details about previous antituberculous therapy were recorded. In all patients with suspected tuberculosis, slides were prepared from three large volume (10-20 ml) sputum samples, Ziehl-Neelsen stained and examined for the presence of acid-fast bacilli. Shortly before this study, large sputum containers (~ 40 ml) had been introduced and patient instruction for sputum collection had been intensified in order to try to increase the diagnostic yield of sputum microscopy. Chest X-rays were examined by an experienced pulmonologist and evaluated for disease extension and the presence of infiltrates, cavities, miliary disease, pleural effusion and fibrotic lesions.

When tuberculosis was confirmed by sputum microscopy or culture of MTB, a standard four-drug regimen (INH, rifampicin, pyrazinamide, and ethambutol) was prescribed [10]. Patients were provided with free and weekly-supervised treatment. During follow-up, physical complaints, body temperature and weight were recorded and sputum was examined at weekly intervals. Chest X-rays were repeated after two and six months. Standard criteria were used for classification of patients and evaluation of therapy [1, 11]. Liver transaminases were measured before and after one and two months of treatment, and HIV-seroprevalence was determined in all patients. Chest X-rays were re-evaluated independently by three experienced clinicians after completion of the study. The study was conducted with informed consent from all patients and with permission from the University of Indonesia.

microbiology

At the time of this study, sputum culture for MTB was not performed in this clinic. For this study, sputum samples were stored at 4^{o} C in an equal volume of cetylpyridinium 0.5%, NaCl 1%, and transported twice-weekly to a reference laboratory for tuberculosis. Culture of *Mycobacterium tuberculosis* (MTB) was performed in 3% Ogawa`s medium. Drug susceptibility testing for INH, rifampicin, ethambutol and streptomycin was done using a proportional method [12]. More than 90% of strains were transported to the Netherlands for susceptibility testing at the National Reference Laboratory for Mycobacteriology, Institute of Public Health and the

Environment (RIVM), a supranational reference laboratory. Here, serial dilutions of five antituberculous drugs on Middlebrook's medium were used [12]. The minimal inhibitory concentration to define drug-resistance were: INH 0,2 mg/L, rifampicin 1 mg/L, ethambutol 5 mg/L, streptomycin 5 mg/L and pyrazinamide 50 mg/L. To confirm drug resistance patterns in this population, 68 additional isolates from non-selected patients presenting at the same clinic were examined at the supranational reference laboratory in the Netherlands.

statistics

Unless stated otherwise, continuous variables are represented as median (interquartile range). Mann-Whitney U test was used for comparison of continuous variables, and Pearson chi-squared test for proportions. All reported *P* values are two-sided, and the level of significance was set at $P < 0.05$.

Results

diagnosis

Based on clinical presentation and chest X-ray, tuberculosis was suspected in 121 patients. Repeated sputum microscopy identified 97 patients (80%) with smear-positive tuberculosis. In comparison, before the introduction of larger sputum containers and intensified instruction of health personnel and patients in the clinic, microscopy was positive in approximately 25% of patients with suspected tuberculosis. The diagnosis was established with the first sputum sample in 81 (84%), with the second sample in an additional 15, and with the third sample in one patient. Sputum cultures, performed in 114 individuals, were positive for MTB in 85 (75%), negative in 28 and contaminated in one. In smear-negative patients, sputum cultures were positive for MTB in three cases, bringing the total number of patients with sputum smear- or culture-positive tuberculosis at 100, or 83% of all suspected cases. Culture remained negative in 12 out of 95 smear-positive patients.

clinical presentation

Patients with bacteriologically proven tuberculosis were mostly young adults, presenting after several months of cough, shortness of breath, chest pain, fever and weight loss (**Table 1**). Symptoms had lasted less than one month in six patients (6%), and one year or more in 16 patients (16%). Fifty-one patients (51%) suffered from moderate to severe malnutrition (BMI < 17). Chest X-ray examination in 100 patients revealed pulmonary infiltration in 92%, cavities in 50%, miliary disease in 5%, pleural effusions in 16%, and old fibrotic lesions in 48%. Bilateral disease was present in 74% patients, and 22% showed abnormalities in all lungfields.

Table 1. Patient characteristics (n=100).

Men / women (%)	57 / 43
Age (yr)	29 (23 – 41)
Duration of symptoms (mo)	3 (2 – 6)
Cough (%)	100
Hemoptysis (%)	38
Dyspnea (%)	77
Chest Pain (%)	72
Fever (%)	60
Night sweats (%)	68
Reported weight loss (kg)	5 (1.8 – 10)
Body weight (kg)	43.3 (36.6 – 47.7)
Body Mass Index (kg/m^2)	16.9 (15.3 – 18.7)
Smoking (%)	35
Previous anti-TB treatment (%)	25
< 1 yr before presentation	14 / 25 (56%)
full course *	2 / 25 (8%)
insufficient regimen **	19 / 25 (76%)
TB in family or household (%)	12

Continuous variables are presented as median (interquartile range).
* 2HRZE,$4H_3R_3$ ** too short (< 5 months), or too limited (< 3 drugs);
see section *'clinical presentation'*

Twenty-five patients had previously been treated for tuberculosis, 14 (56%) within 1 year before presentation (**Table 1**). Only two patients (8%) had been prescribed adequate treatment for 6 months (2HRZE, $4H_3R_3$), while 19 (76%) had received insufficient treatment. In 14 patients the duration of treatment had been too limited (range: 1-5 months). In an additional 5 patients, monotherapy with rifampicin (n = 2) or pyrazinamide (n = 1), or treatment with two drugs only (n = 2) had been prescribed. In four patients, no data were available about the nature and length of previous antituberculous treatment.

HIV-infection

HIV-infection was found in one patient (1%), who presented with classical symptoms of tuberculosis and a chest X-ray showing widespread infiltration and a small cavity in the left upper lobe. Sputum microscopy and MTB culture were positive, no drug-resistance was found, and the response to treatment was unremarkable.

drug resistance

Drug susceptibility testing of patient isolates was performed in two laboratories. At the reference laboratory in the Netherlands, 55 out of 78 (70.5%) strains examined were fully susceptible, while 23 (29.5%) showed resistance against one or more antituberculous drugs. Susceptibility testing of 68 additional patient isolates from the same clinic showed similar results. In **Table 2**, overall resistance and individual patterns of all 146 strains are presented. Resistance was found to all first-line drugs including pyrazinamide. Nine strains (6.1%) were resistant to at least rifampicin and INH (multidrug resistance). At the WHO collaborative laboratory in Indonesia, oyrazinamide is not included in susceptibility testing. In addition, in comparison with the 78 strains tested at the supranational reference laboratory in the Netherlands, resistance to ethambutol (n=4) was missed in three patients, and monoresistance to INH (n=9) in one patient. On the other hand, resistance to streptomycin was falsely diagnosed in six patients, and multidrug resistant tuberculosis in two.

Drug resistance (as established in the supranational reference laboratory) was higher in retreatment cases than in new cases (39.5% vs 25.9%; *P* =0.08). Among retreatment cases, many were resistant to drugs they had never been treated with (data not shown). Out of nine multidrug resistant cases, five had never been treated for tuberculosis and one (who was resistant to all five drugs tested) had been prescribed a full course of antituberculous drugs in 1991.

treatment

Early in the course of treatment, one patient was referred to hospital with severe hemoptysis, and one patient died. The majority of patients however, improved within weeks. Fever abated after one or two weeks and most complaints resolved within four to six weeks of therapy. After two months, 70 out of 88 patients evaluated (80%) had gained a median of 2.5 kg of weight (range: 0.5 - 9), while 16 (18%) had lost 1.1 kg (range: 0.8 – 2.1). The fasting month may have contributed to weight loss in some patients.

Many patients suffered from mild and transient side effects of the antituberculous medication. Among 95 patients evaluated, only four males and one female remained without side effects during the first two months of treatment. Out of 95 patients evaluated, 62 (65%) reported gastrointestinal complaints, while 63 patients (66%) complained about painful joints, and 67 patients (71%) about itching. Blurred vision and color blindness, a possible side effect of ethambutol, was present in five patients. One patient developed a peripheral neuropathy. Hepatotoxicity was not established: neither jaundice, nor a three-fold rise in liver transaminases was found.

Table 2.
Drug resistance of MTB isolates *

	new cases (n=108)	retreatment cases (n=38)	all cases (n=146)
Overall resistance			
INH	23 (21.3%)	13 (34.2%)	36 (24.7%)
rifampicin	5 (4.6%)	4 (10.5%)	9 (6.1%)
any first-line drug	28 (25.9%)	15 (39.5%)	43 (29.5%)
Resistance patterns #			
HRZES	0	1	1
HRES	1	0	1
HRS	2	1	3
HR	2	2	4
HES	1	0	1
HE	1	1	2
HS	4	1	5
H	12	7	19
Z	2	1	3
S	3	1	4
fully susceptible	80	23	103

* resistance patterns from 78 isolates from the original patient cohort and 68 additional non-selected isolates were combined (see: methods). # H= INH, R= rifampicin, Z= pyrazinamide, E= ethambutol, S= streptomycin.

outcome

The overall cure rate, mostly based on documented sputum conversion, was 75% (**Table 3**). Due to difficult expectoration of sputum, bacteriological evaluation at 6 months was limited. The proportion of patients that defaulted was 10%. No significant differences were found between non-adherent and adherent patients with regards to age, sex, disease severity, and distance to the outpatient clinic (data not shown). During six months of treatment, seven patients (7%) died (three females, four males; age: 18 – 46 years). Four suffered from respiratory failure, two from massive hemoptysis, and one patient reportedly died with “high fever”. At presentation, chest X-rays had revealed extensive cavitary disease in four, widespread pulmonary

infiltrates in two, and a destroyed lung in one patient. Patients who died had a lower BMI at presentation than patients who survived (14.7 versus 17.0; *P* = 0.02). Drug resistance had not been detected in any of the patients who died. One patient who died had received an incomplete course of antituberculous drugs six months before presentation, while six others had never been treated before. All patients who died had initially shown a positive response to treatment, and all had gained some weight.

Table 3.
Treatment outcome in 100 tuberculosis patients *

Cured / completed treatment	75	
Treatment defaulted	10	
< 2 months		7
> 2 months		3
Incomplete treatment	2	
Treatment failure	3	
Transfer out	3	
Death	7	
< 2 months		1
> 2 months		6

* *Cured*: negative sputum smear on 2 occasions after treatment. *Completed treatment*: negative sputum smear 2 months after start of treatment, with no or only 1 negative sputum smear after treatment completion. *Treatment defaulted*: < 4 mths of treatment. *Incomplete treatment*: between 4 and 5 months of treatment, with negative sputum smear received 2 months after start of treatment. *Treatment failure*: > 5 months of treatment, with signs of active disease including sputum smear positive results > 5 months after start of treatment. *Transfer out*: transferred to another clinician. *Death*: death, irrespective of cause.

Discussion

This report presents a detailed description of a cohort of Indonesian patients with tuberculosis. In the current TB-epidemic in Indonesia, little information is available with regard to important clinical aspects like quality of diagnosis and treatment, prevalence of HIV-infection, and presence of drug resistant MTB isolates. The size of the study population and the fact that it was included at a single outpatient clinic, limit the generalizability of the results. However, although our findings may not be representative, health workers in Indonesia and elsewhere may face similar difficulties while caring for the large number of patients with suspected tuberculosis.

The majority of patients in this cohort presented with classical symptoms of pulmonary tuberculosis. Cough was present in all patients, and weight loss was very common. Also, hemoptysis and shortness of breath, signifying extensive parenchymal involvement, were often present. Ninety-four percent of patients presented after more than one month of symptoms. HIV-seroprevalence was low (1%), in accordance with another recent survey in the Infectious Disease Hospital in Jakarta, in which two out of 99 tuberculosis patients were HIV-positive (Dr. R.H.H. Nelwan, personal communication).

Accurate diagnosis is important for control of tuberculosis. Unfortunately, in Indonesia antituberculous drugs are often prescribed without bacteriological confirmation of tuberculosis. Because of this, some (infectious) patients will remain untreated, while others not suffering from tuberculosis will unnecessarily receive precious medication. In light of the very large number of patients with suspected tuberculosis, any proportion of false positive diagnosis will have serious health economic consequences. In this report it was shown that microscopy of two large sputum samples was a very strong and cost-effective diagnostic tool, with high sensitivity and specificity for tuberculosis. This high diagnostic yield of sputum microscopy is a remarkable finding. Obviously, in an endemic setting, patients have a high risk of suffering from active tuberculosis. A recent report showed that sputum microscopy may be 80% sensitive in patients with a high (> 75%) clinical suspicion of tuberculosis [13]. In addition, we believe that introduction of large sputum containers and intensified instruction shortly before this study may have led to an increased number and better quality of sputum samples collected. Sputum culture rarely became positive in smear-negative cases and remained negative in 12% of smear-positive patients. This may have been due to the decontamination procedure used or to the fact that only a single culture was done for every patient.

In this cohort, drug resistance was found against all first-line drugs with 25% of isolates resistant to INH, and 6% to rifampicin. Testing of isolates from 68 additional,

non-selected patients from the same clinic confirmed these findings. These figures are high, but still lower than reported in a hospital survey (Annual Report St Carolus Hospital, 1994) and by the WHO-collaborative laboratory: both showed > 30 % resistance against INH and > 10 % against rifampicin [7]. Significant differences were found between results from the local laboratory and a supranational reference laboratory. For technical and economic reasons, culture and drug susceptibility testing of MTB isolates is very limited in Indonesia. Proficiency testing or supranational supervision is not routine.
In Indonesia, the majority of patients is treated outside the national program. Of our patients, 25% had previously received antituberculous treatment. Most drug regimens had been inadequate, which may have contributed to the development of drug resistance. However, one out of every four patients not previously treated for tuberculosis was infected with a drug resistant strain. Among patients with recurrent disease, we were unable to correlate previous treatment regimens with drug resistance patterns. Therefore, recurrent disease in this highly endemic setting may result from exogenous reinfection rather than from relapse [14].

Several observations were made with regard to treatment. Firstly, even though the dosage of antituberculous drugs was not adjusted for body weight in these malnourished patients and side-effects were common, hepatotoxicity was absent in this study cohort. Insufficient compliance during weekly-supervised treatment provides a possible explanation. Alternatively, drug absorption is reduced, metabolism is altered, or quality of locally produced drugs is sub-optimal. Further investigations are necessary to elucidate this issue. Second, the default rate (10%) in this cohort, although much lower than the WHO-reported national average, leaves room for improvement. Within this small cohort, we were unable to identify patient characteristics related treatment default. Third, with respect to treatment outcome, the single most important risk factor for death was malnutrition. Protein-energy malnutrition and micronutrient deficiencies, which may both affect host defense against tuberculosis, are common among Indonesian tuberculosis patients [8]. Supplementation of vitamin A and zinc had beneficial effects in Indonesian patients [15].

In summary, this explorative study provides useful information about various clinical aspects of tuberculosis in Indonesia. With regard to diagnosis, sputum microscopy is a very sensitive and specific diagnostic tool in this endemic setting. This may be due to the fact that disease was longstanding, but we think that collection of large and good quality sputum samples may help to increase the case-detection rate and to limit false-positive diagnosis and unnecessary prescription of antituberculous drugs. The observed drug resistance patterns warrant the implementation of more extensive and reliable susceptibility testing. HIV-infection is still rare in this setting, but

malnutrition is common, and nutritional interventions may benefit treatment outcome. Data on previous treatment regimens and the current default rate led us to conclude that at least in this urban setting, many Indonesian patients receive insufficient or incomplete drug therapy. All together, although our findings may not be representative, they highlight some of the problems of clinical management of tuberculosis patients in Indonesia.

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Chapter 9

A simple intervention to increase the yield of sputum microscopy for tuberculosis in a resource-poor setting in Indonesia

Bachti Alisjahbana[1], Reinout van Crevel [2], Halim Danusantoso [3], Tintin Gartinah [4], Eddie S. Soemantri [1], Ronald H.H. Nelwan [5], Jos van der Meer [2].

[1] Department of Internal Medicine, Faculty of Medicine, Padjadjaran University, Bandung, Indonesia. [2] Dept. of Medicine, UMC Nijmegen, The Netherlands, [3] Indonesian Tuberculosis Association, Jakarta Branch, [4] West Java Provincial Referral Laboratory, [5] Working Group on Infectious Diseases, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia.

submitted

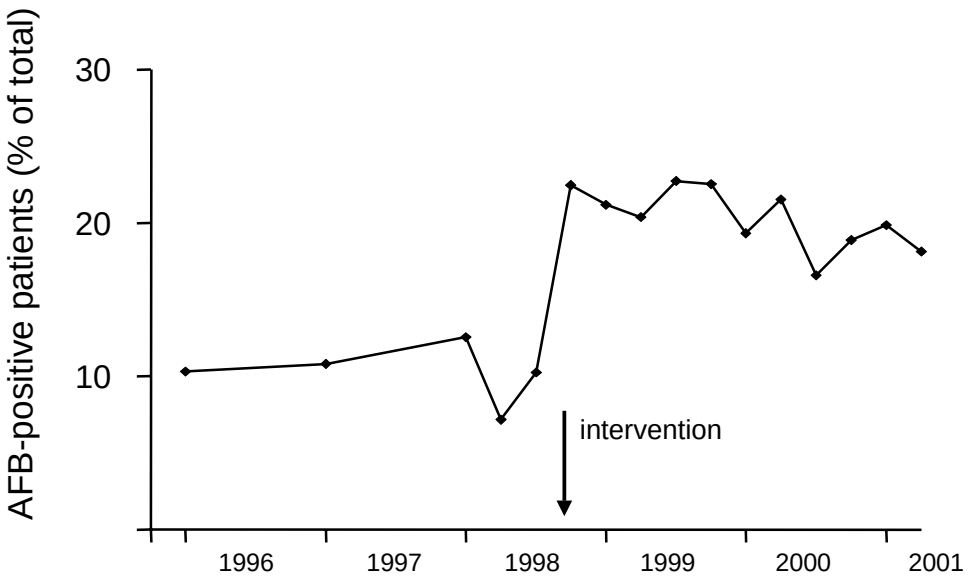
Abstract

In many resource-poor settings, low yield of sputum microscopy may contribute to insufficient TB case-detection. The number and quality of sputum samples provided by the patient may be important in this respect. After introduction of larger sputum containers and education of health staff and patients, we observed a sustainable two-fold increase of the smear-positivity rate in an urban clinic in Indonesia during three years follow-up. This effect was confirmed in a randomized controlled study; brief counselling by paramedics resulted in a substantial increase in positive samples, number of acid-fast bacilli per sputum slide, and patients diagnosed with smear-positive TB. The effect of education was especially pronounced in female patients. Simple interventions like these may increase TB case-detection in Indonesia and elsewhere.

Microscopic detection of acid-fast bacilli (AFB) in sputum is the mainstay for diagnosing pulmonary tuberculosis (TB). According to the textbooks, the sensitivity of sputum microscopy for TB is 60-80%. Unfortunately, in resource-poor settings with a high burden of TB, case-detection is often much lower [1]. This is probably caused by low access to health service and insufficient quality of sputum microscopy. In addition, the number and quality of sputum samples provided by the patient may be important [2]. We evaluated the effect of education in combination with large sputum containers on the yield of sputum microscopy for TB in two urban clinics in Indonesia.

In September 1998, an observational study was started to describe the clinical and microbiological status of patients in a TB-control clinic in a poverty area of Jakarta, Indonesia. Patients in this clinic usually present with longstanding and severe disease. However, at that time, many patients with extensive radiographic abnormalities remained sputum smear (AFB)-negative. In this resource-poor setting, the staining method was used correctly, the microscopist was well trained and the microscopes were functioning well. However, we noted that very small containers were used for sputum collection and that many patients provided saliva rather than sputum. In an effort to increase case detection, larger sputum containers were introduced. In addition, health staff from then onwards instructed the patients to provide bigger samples of early-morning sputum. Within weeks, the number of AFB-positive patients increased substantially. Since then, with no further adjustments to the diagnostic process, case detection has remained approximately two-fold higher than before (see **Figure 1**).

To examine the role of patient education more closely, patients with newly suspected tuberculosis were randomly assigned to a control or an intervention group in an outpatient TB-clinic of a general hospital in Bandung, Indonesia. Patients in the control group followed the routine procedure for sputum collection. Patients in the intervention group were addressed individually by a paramedic, who briefly explained about the importance of sputum examination and instructed the patient how to produce adequate samples. Large clear sputum containers (diameter 5 cm, height 7 cm) were used in both groups. Microscopy of sputum smears was performed by laboratory technicians who were unaware of the patient's identity or study group.



patients examined (no.)	*2865*	*2491*	*2054*	*3256*	*3229*	*1785*

Figure 1.

AFB (acid-fast bacilli)-positive patients as % of the total number of patients examined in an urban TB-clinic in Jakarta, Indonesia (January 1996 – July 2001). Since September 1998, larger sputum containers are used, and patients are instructed to provide good-quality sputum. Since this time, 1792 out of 8858 patients have been found AFB-positive (20.23%), compared with 706 out of 6822 patients (10.34%) in the previous 3-year period (*P < 0.001*)

We randomized 110 patients with suspected TB (57 males and 53 females, 35.5 +/- 17.3 years of age). The control (n=60) and intervention group (n=50) were similar in terms of duration of disease, symptoms and chest X-ray abnormalities (data not shown). A higher sputum smear-positive TB rate was found in the intervention group ; 22 patients of the intervention group (44%) compared with 18 (30%) of patients in the control group were AFB positive (*NS).* Patients provided a total of 268 sputum samples; 141 for the control group and 127 for the intervention group. In the intervention group, the volume, purulence and leucocyte count of sputum samples was significantly higher (data not shown). In addition, the smear-positive rate (number of positive samples / number of samples examined) and density of AFB were higher (**Table 1**). Fifty-six out of 127 (44%) samples in the intervention group were AFB-positive compared with 40 out of 141 (28%) in the control group ($P < 0.01$). Interestingly, smear-positive rate and effect of education were different between males and females. In females, 26% of specimens in the control group were positive, versus 58% in the intervention group ($P < 0.001$). In males this difference was not so profound (33% vs. 39%, *NS*).

The number of patients examined in this controlled study limits its statistical power. However, the main conclusion of this trial is very much supported by evaluation of 6822 patients before, and 8858 patients after a simple intervention in a TB-clinic as presented in **Figure 1**. Therefore, we conclude that simple means to improve volume and quality of sputum samples may result in a substantial and sustainable increase of the diagnostic yield of sputum microscopy for TB in a high-burden setting. Interestingly, the effect of education seems to be much larger in females. Previous reports have shown a lower case-detection of TB in females [3]. In South East Asia, the estimated female / male ratio is 0.3 [4]. This is probably caused by a lower disease prevalence in women [4]. Our study indicates that quality of sputum samples may also contribute to this difference and that education may help to improve diagnosis in female patients with suspected TB. We hypothesise that for socio-cultural reasons, women in Indonesia may be reluctant to provide purulent sputum. Implementation of simple and cheap interventions like these may contribute to early diagnosis and treatment of TB, which will benefit both the individual patient and the community.

Table. 1

Characteristics of sputum samples provided by patients randomly assigned to the routine procedure (n=60) or educational intervention (n=50) in a general hospital in Bandung, Indonesia. The IUATLD scoring-system was used to quantify the AFB.

	Control group	Intervention group
Number of samples	141	127
Samples / patient (no.)	2.35 +/-	2.54 +/-
Density of AFB on sputum smear *		
0	101 (71.6%)	71 (55.9%)
+	29 (20.6%)	26 (20.5%)
++	6 (4.3%)	17 (13.4%)
+++	5 (3.5%)	10 (7.8%)
Any positive *	40 (28.4%)	56 (44.1%)

* significantly different between groups; $P < 0.05$ (Fisher exact-test, two-sided)

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Chapter 10

Mycobacterium tuberculosis Beijing genotype strains are associated with a febrile response to treatment

Reinout van Crevel[1], Ron H.H. Nelwan [2], Wilma de Lenne [1], Yelilselvan Veeraragu [4], Adri G. van der Zanden [5], Zulkifli Amin [3], Jos W.M. van der Meer [1], Dick van Soolingen [6]

[1] Dept. of Medicine, University Medical Center Nijmegen, The Netherlands.[2] Working Group on Infectious Diseases, [3] Division of Pulmonolgy, Dept. of Internal Medicine, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia.[4] Trisakti University, Jakarta, Indonesia. [5] Dept of Medical Microbiology, and Infectious Diseases, Gelre Hospital, Apeldoorn, The Netherlands. [6] National Reference Laboratory for Mycobacteriology, National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

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Abstract

DNA fingerprinting has demonstrated predominance of the ‘Beijing’ genotype among Mycobacterium tuberculosis strains isolated in South East Asia. We prospectively examined the occurrence of Beijing genotype strains in tuberculosis patients in Indonesia. Of the 93 *M. tuberculosis* isolates, 32 (34%) were of the Beijing genotype. Beijing strains were more often resistant to INH (36% vs. 20%) and streptomycin (15% vs. 5%), but the rate of multi-drug resistance was low. No significant associations were found between the *M. tuberculosis* genotype and patient’s age, BCG vaccination status or clinical presentation. However, within the first weeks of treatment, patients infected with Beijing genotype strains more often (48% vs. 21%; *P* = .02) developed a febrile response, which was not related to disease severity, toxicity or drug resistance. This finding indicates that Beijing genotype strains may have specific pathogenic properties, possibly contributing to their spread.

Introduction

In 1995 DNA fingerprinting has shown that the vast majority (over 80%) of a collection of *Mycobacterium tuberculosis* isolates from China belonged to a genetically closely related group of bacteria, designated the “Beijing genotype family” [1]. Strains of this genotype family were also found to dominate in neighboring countries in South East Asia, whereas a lower prevalence was found on other continents [1]. Recently, it was shown that 50% of the 563 isolates from Vietnam belonged to the Beijing genotype and, moreover, that the occurrence of these strains correlated significantly with young age and, hence, with active transmission of tuberculosis [2]. It was speculated that strains of the Beijing family recently expanded from an evolutionary lineage with an unknown selective advantage over other *M. tuberculosis* genotypes [1]. ‘Strain W’, a highly drug-resistant strain which caused large nosocomial outbreaks in New York City in the early 90’s [3,4], is an evolutionary branch of the Beijing genotype family [5].

Worldwide, Indonesia has the third highest number of tuberculosis patients with an estimated 591.000 cases in 1998 [6]. No data have been published from Indonesia on the distribution of *M. tuberculosis* genotypes. We prospectively collected demographic and clinical data and performed DNA fingerprinting of *M. tuberculosis* isolates from a cohort of Indonesian patients in Jakarta. This enabled us to assess the prevalence of the Beijing genotype strains, and to compare drug-resistance and clinical course of patients infected with Beijing- and other genotype strains of *M. tuberculosis.*

Methods

Patient inclusion and follow-up

From December 1998 through March 1999, 121 consecutive patients were included at the Perkumpulan Pembertasan Tuberkulosa Indonesia (PPTI), an outpatient tuberculosis clinic in a densely populated area in Jakarta. Demographic data, symptoms and signs, risk factors for tuberculosis, and details about previous antituberculous therapy were recorded. From three large volume sputum samples, microscopy for acid-fast bacilli (AFB) and culture for *M. tuberculosis* were performed. Chest X-rays were made before start of treatment and evaluated by two experienced pulmonologists. HIV-seroprevalence was determined. Informed consent for the investigations was obtained from all patients. When tuberculosis was bacteriologically confirmed, a standard four-drug regimen (INH, rifampicin, pyrazinamide and ethambutol) was prescribed [7]. During follow-up, physical complaints, body temperature and weight were recorded.

Culture of mycobacteria and drug susceptibility testing

After standard processing of sputum samples, culture was performed in 3% Ogawa`s medium. Twice weekly, slants were examined for the appearance of colonies. At the National Institute of Public health and the Environment (RIVM), Bilthoven, the Netherlands, susceptibility testing of patient isolates was done using serial dilutions of five antituberculous drugs on Middlebrook's medium [8]. The minimal inhibitory concentration used to define drug-resistance were: INH 0,2 mg/l, rifampicin 1 mg/l, ethambutol 5 mg/l, streptomycin 5 mg/l, and pyrazinamide 50 mg/l.

DNA fingerprinting and spoligotyping

Genotyping of mycobacterial isolates was done using restriction fragment length polymorphism (RFLP) typing. Extraction of DNA from *M. tuberculosis* strains and Southern blotting with labeled IS*6110* DNA as a probe were done by standard DNA fingerprinting method [9]. Spacer oligonucleotide typing (spoligotyping) of *M. tuberculosis* DNA from patient isolates was done as previously described [10]. From culture-negative patients, *M. tuberculosis* DNA for spoligotyping was directly isolated from sputum smears [11]. Isolates were classified as Beijing genotypes when a hybridization reaction was found on the last nine spacers (35-43) in spoligotyping [1]. The computer-assisted analysis of the IS*6110* fingerprints was done with the Windows version of Gelcompar (Applied Maths, Kortrijk, Belgium).

Statistics

Descriptive results for continuous variables are reported as median (range), and for categorical data as percentages. Mann-Whitney U test was used for comparison of continuous variables, and Pearson chi-squared test for proportions. All reported P values are two-sided, and the level of significance was set at $P < .05$.

Results

Genotyping and susceptibility testing of M. tuberculosis strains

In 121 consecutive patients with a clinical diagnosis of tuberculosis, direct examination of at least one sputum smear was positive for AFB in 89 patients (73%), and *M. tuberculosis* was cultured from 84 of 113 single cultures performed (75%). **Figure 1** represents the IS*6110* restriction fragment patterns of these samples. The vast majority (85%) of strains demonstrated a fingerprint pattern not found in other strains included in this study. Twenty-eight strains exhibited a high number of IS*6110*-containing restriction fragments and a very high degree of similarity (> 66%). This homogenous group of isolates represented the Beijing family of genotypes, as was confirmed by spoligotyping **(Figure 1)**. Direct spoligotyping on sputum smears of culture-negative patients added another four patients with Beijing strain infections. All together, from a total number of 93 *M. tuberculosis* strains analyzed (84 cultured isolates, using IS*6110* RFLP and spoligotyping; and 9 stained sputum smears, using spoligotyping only), 32 strains (34%) were Beijing genotypes.

Cultures became positive for *M. tuberculosis* after 4.7 weeks for Beijing strains compared with 5.2 weeks for non-Beijing strains (NS). Drug susceptibility testing revealed a trend towards a higher prevalence of resistance to INH (36% vs. 20%; $p = .09$) and streptomycin (15% vs. 5%; $p = .16$) in Beijing strains compared with non-Beijing strains (**Table 1**). There was no significant difference between the two groups in the prevalence of multidrug resistance (7% vs. 4%). Both for Beijing and non-Beijing strains, drug resistance was equally found among different age groups (data not shown).

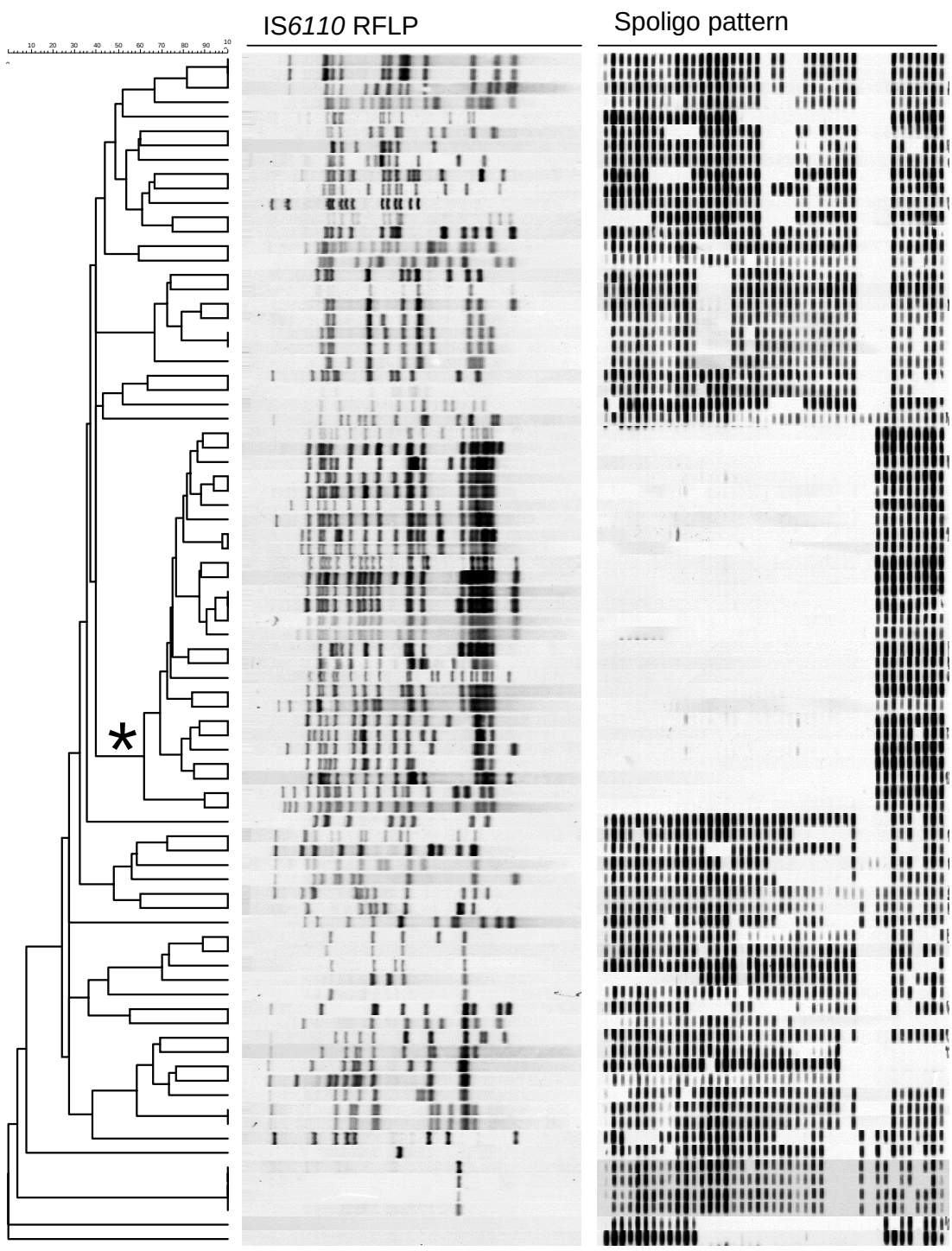

Figure 1.

Dendrogram showing similarity of the 84 IS*6110* RFLP patterns of *M. tuberculosis* isolates from Jakarta, in combination with the respective spoligo patterns.

The branch in the dendrogram representing Beijing genotype isolates is indicated with an asterix (*).

Comparison of patients according to M. tuberculosis genotype

The characteristics of patients infected with Beijing genotypes (n=32) were compared to the ones of patients infected with other strains (n=61) (**Table 1**). The age distribution in the two groups was similar, although no patients under the age of 16 were evaluated. No relation was found between genotype and BCG vaccination status either (**Table 1**). Of the patients infected with Beijing strains, 7 (22%) had been using one or more antituberculous drugs before, compared to 18 (29%) of patients infected with non-Beijing strains. Only two patients (one in either group) had been prescribed a standard regimen for 6 months. At time of presentation, there were no significant differences in the presence of fever, dyspnea or hemoptysis, or the duration of symptoms (data not shown). The nutritional status in both groups of patients was similar as judged by body mass index (17 vs. 16.9 kg/m^2). The smear-positivity was equally high in both groups, and the density of acid-fast bacilli in sputum smears was similar (data not shown). Radiographic evaluation of patients did not reveal significant differences either; the number of lungfields involved was similar and an equal percentage in both groups presented with pulmonary cavities (**Table 1**). Only one patient in this cohort, presenting with cavitary tuberculosis, appeared HIV-positive; this patient was infected with a Beijing strain.

Patients were evaluated at weekly intervals. The majority of patients demonstrated an early and beneficial response to treatment. In both groups, 20 % continued to lose weight during the first two months. Body weight increased a median of 2 kg (range: 0 – 8 kg) in the remaining patients in both groups. No relation was found between drug resistance and changes in body weight. At six months patients in both groups had equally gained weight (median: 5 kg). No active disease was established at this point, but treatment was extended in patients with multiple drug resistance.

Thirty two percent of the patients developed fever (> 38^0 C, maximum 39.3^0 C) during the first weeks of treatment. No patient reported shaking chills during this period. Interestingly, this transient febrile response, which lasted 2 to 3 weeks, was found in 15 out of 31 patients (48%) infected with Beijing strains compared with 13 out of 61 patients (21%) with non-Beijing strains ($p = .02$). Drug resistance could not explain this finding: 46% of patients infected with a fully susceptible Beijing strain compared with 19% of patients infected with susceptible non-Beijing strains showed a febrile response ($p = .06$). Disease severity did not account for this difference either as there was no association between the febrile response and the nutritional status, or between fever and the presence of pulmonary cavities (data not shown). Drug-toxicity, which may also induce fever, was not established.

Table 1.
Drug resistance and patient characteristics according to *M. tuberculosis* genotype.

	Beijing (n=32)	non-Beijing (n=61)
Male	19 (59%)	35 (57%)
Age (years)	31 (19-68)	29 (17-70)
BCG-scar	8 (25%)	14 (23%)
Chest X-ray [1]		
Cavities	16 (50%)	29/55 (53%)
Bilateral disease	25 (78%)	40/55 (73%)
Patient strain resistant to [2]		
INH	10 / 28 (36%)	11 / 54 (20%)
Rifampicin	2 / 28 (7%)	2 / 54 (4%)
Pyrazinamide	0	3 / 54 (5%)
Ethambutol	1 / 28 (4%)	3 / 54 (5%)
Streptomycin	4 / 28 (14%)	3 / 54 (5%)
Any prescribed drug	11 / 28 (39%)	14 / 54 (26%)
Febrile response to treatment *	15 / 31 (48%)	13 / 61 (21%)

[1] Six chest X-rays in the non-Beijing group were only evaluated by a single pulmonologist.
[2] In both groups, 88% of patients was culture-positive, providing 28 respectively 54 isolates for susceptibility testing. * Significant difference between groups (Mann-Whitney-U test; $P = .02$).

Discussion

Worldwide, DNA fingerprinting has revealed extensive heterogeneity of *M. tuberculosis* genotypes [12]. However, a distinct and predominant group of *M. tuberculosis* genotypes, termed Beijing, has been found in the People's Republic of China and neighboring countries [1,13]. The present study indicates that Beijing strains are also present in the Indonesian archipelago.

The prevalence of Beijing strains found in China (85%), Mongolia (50%), South Korea (43%), Thailand (37%), Vietnam (50%) and Indonesia (34%) suggests that this clone spreads in South East Asia, where tuberculosis is endemic. However, also in former USSR republics Azerbaijan and Estonia [14,15], and in Cuba [16], the Beijing genotype strains account for a significant part of tuberculosis cases involving (multidrug) resistance. Strain W, which also represents the Beijing genotype family [5], caused outbreaks of MDR-TB during the past decade in the United States [3,4] and in South Africa [17]. In summary, all reports on the occurrence of Beijing genotypes show a clear correlation with drug resistance. In our study, 36% of the Beijing strains was resistant to INH. However, multi-drug resistance was limited, which makes it unlikely that drug resistance is the single explanation for the predominance of Beijing strains in this population.

Different transmission rates may also account for an unequal distribution of genotypes. In a tuberculosis outbreak in the United States, a particular *M. tuberculosis* genotype caused extensive transmission, as evaluated by skin test conversion [18]. In Indonesia, outbreak investigations like these seem impossible in light of the high prevalence of tuberculosis, and standard BCG vaccination that hampers the interpretation of tuberculin skin tests. However, indirect evidence supporting an increased transmission of Beijing strains comes from a recent study in Vietnam, which demonstrated that Beijing strains were more prevalent among young patients [2]. We could not confirm this in the Indonesian patients, but we did not investigate patients under 16 years of age. In agreement with the study in Vietnam, we did not find a correlation between vaccination status and genotypes.

Disease severity in patients infected with Beijing or non-Beijing genotypes seemed similar. However, our prospective evaluation revealed a different response to treatment. In 48% of the patients infected with Beijing strains and 21% of the other patients a transient febrile response was found shortly after start of antituberculous treatment. Neither disease severity nor drug toxicity or drug resistance accounted for this difference. The increased febrile response in patients with Beijing strains is remarkable and suggests that Beijing strains elicit a different host response. Although the number of isolates limits the power of this study, it is tempting to speculate that

this is relevant to the distribution of Beijing genotypes. There may be an interesting parallel with the outbreak in the United States, as mentioned above [18], since the causative strain in that outbreak, designated CDC1551, induced a more rapid and robust in-vitro production of pyrogenic cytokines like interleukin-6 and tumor necrosis factor-α [19]. It remains to be determined whether Beijing strains also elicit a different cytokine response in animal models or patients.

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Chapter 11

Low plasma concentrations of rifampicin in tuberculosis patients in Indonesia.

Reinout van Crevel[1], Bachti Alisjahbana[2], Wiel de Lange[3], Filip Borst[1], Halim Danusantoso[4], Jos W.M. van der Meer[1], David Burger[5], Ronald H.H. Nelwan [6]

[1] Dept of Internal Medicine, [3] Dept of Pulmonary Medicine, [5] Dept of Clinical Pharmacology, University Medical Center Nijmegen, the Netherlands. [2] Dept of Internal Medicine, Padjadjaran University, Bandung, Indonesia, [4] Perkumpulan Pemberantasan Tuberkulosa Indonesia (PPTI), Jl Baladewa, Jakarta, Indonesia. [6] Working Group on Infectious Diseases, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia

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Abstract

Rifampicin is a key drug in tuberculosis treatment, yet little is known about its quality and bioavailability in countries endemic for tuberculosis. High drug levels may lead to increased toxicity, while low drug levels may predispose for treatment failure and relapse. We measured plasma concentrations of rifampicin among 62 nonselected tuberculosis patients in Jakarta, Indonesia and tested the rifampicin content of the drug formulations in use. Plasma concentrations were related to patient characteristics, drug manufacturer, toxicity, treatment outcome and in vitro susceptibility of patient *M. tuberculosis* isolates. The plasma concentrations of rifampin were generally low: 70% of patients had two-hour plasma concentrations (C_{max}) below 4 mg/L. No toxic plasma concentrations of rifampicin (> 20 mg/L) were found. The strongest predictive factor for the magnitude of rifampicin concentrations was the drug manufacturer. The rifampicin content of the different drug preparations used was between 90.5 and 103.6% of the reference standard. The median ratio C_{max} / MIC was 9.8 (range: 0 - 46.8). No association was found between low plasma rifampicin concentrations and delayed sputum conversion or treatment failure. The unexpectedly low plasma concentrations of rifampicin, which were found in this setting, are most likely due to reduced bioavailability of local drug preparations, as rifampicin content of the drug preparations was normal.

Introduction

Because of its strong antimycobacterial capacity, rifampicin is necessary for successful first-line antituberculous treatment [1]. Little is known about its bioavailability and pharmacokinetics in resource-poor countries where more than 95% of the world's tuberculosis patients reside [2]. On the one hand, the low body weight of tuberculosis patients may be associated with higher drug levels and increased drug-toxicity [3]. On the other hand, if absorption of these drugs is impaired [4], or quality of locally produced antituberculous drugs is uncertain [5,6], one may find low drug concentrations. Such low drug concentrations, especially of rifampicin, may predispose to treatment failure or relapse [7,8]. We investigated possible variation of the bioavailability of rifampicin in Indonesia, a country with a high tuberculosis case-load [9], which is strongly associated with malnutrition [10].

Methods

patients

Between September and December 2000, 62 consecutive patients with microbiologically proven pulmonary tuberculosis were investigated in an outpatient tuberculosis clinic in Jakarta, Indonesia. In accordance with the Indonesian national guidelines, treatment consisted of isoniazide (INH) 300 mg, rifampicin 450 mg, pyrazinamide (PZA) 1500 mg and ethambutol 750 mg daily for two months, followed by INH 600 mg and rifampicin 450 mg three times weekly for four months [11]. Treatment was not adjusted for body weight. For every patient, a single box with all medication for six months was kept at the clinic throughout the period of treatment. All patients visited the clinic at weekly intervals, when drug intake was observed by health personnel and medication was provided for the remaining days of the week. Patients received all drugs free of charge. The outcome of treatment was monitored using standard criteria [12]. This study was conducted with informed consent from all patients and with permission from the University of Indonesia.

microbiology

At least two sputum samples were collected for microscopy and culture before treatment, and after two and six months. Culture of *M. tuberculosis* was performed in 3% Ogawa's medium. Drug susceptitbility testing of isolates was done using serial dilutions of antituberculous drugs on Middlebrook's medium [13]. Minimal inhibitory drug-concentrations (MIC) to define antimicrobial resistance were: 0.2 mg/L for INH, 1 mg/L for Rif, 5 mg/L for ethambutol and streptomycin, and 50 mg/L for PZA.

measurement of rifampicin in plasma samples and drug preparations

Patients were evaluated after 4 and 8 weeks of treatment, when stable drug levels were expected. Apart from analgetics and antitussives, no other co-medication was allowed. Patients were asked not to have breakfast on the morning of blood sampling. Witnessed intake of all four antituberculous drugs took place in the clinic between 8.00 and 10.00 a.m., at least 24 hours after previous drug intake. Two hours after drug intake, corresponding with the estimated time to maximum plasma concentrations of rifampicin [14], 10 ml of venous blood was collected. Following immediate centrifugation, plasma was separated and frozen at -20^0 C in polypropylene tubes containing 20 mg/mL ascorbic acid. All samples were stored at -80^0 C within 4 hours. Measurement of rifampicin in plasma samples was done by high performance liquid chromatography [15]. For measurement of rifampicin in drug formulations, two blisters were collected from every individual patient, each containing medication for a single day of intensive treatment. Manufacturer, batch-number and expiration date of medication were recorded. Medication was kept in closed plastic bags at room temperature and protected from sunlight until analysis. For chromatographic measurement of rifampicin content, single tablets were dissolved by addition of MeOH, followed by ultrasonic centrifugation and homogenization.

data analysis

In patients with plasma concentrations of rifampicin available at weeks 4 and 8, the mean value was used for further analysis, unless one value was below 1 mg/L and less than 25% of the second value, in which case the lower value was excluded. Two-hour plasma concentrations (C_{max}) of rifampicin > 20 mg/L were considered toxic, values between 8 and 20 mg/L therapeutic, between 4 and 8 mg/L “low”, and < 4 mg/L subtherapeutic (“very low”)[16]. Plasma concentrations of rifampicin were correlated with gender, body weight, presence or absence of diarrhea, drug manufacturer and HIV-infection. Collected drug preparations of rifampicin were defined as substandard if they contained less than 85% or more than 115% of the reference standard [5]. The possible significance of low plasma concentrations was investigated by calculating the ratio of C_{max} / MIC for rifampicin [17], and by comparing clinical and bacteriological outcome in patients with rifampicin concentrations below and above 4 mg/L.

statistics

Variables are represented as mean (SD) when normally distributed and as median (range) in all other cases. Pearson χ^2-test, Student’s *t*-test, Mann-Whitney test and univariate regression analysis were used as appropriate. Statistical analysis was performed using SPSS version 9.0 for Windows (SPS Inc., Chicago, IL.). All reported *P* - values are two-sided, and the level of significance was set at $P < 0.05$.

Results

Plasma concentrations of rifampicin

Tuberculosis patients included in this study were mostly young adults and more often of male origin (**Table 1**). Moderate or severe malnutrition (body mass index < 17 kg/m^2) was present in 45%.

Table 1.

Patient characteristics

Male gender	43 (69%)
Age (yrs)*	34 (16 - 62)
Sputum microscopy	
+	18
++	32
+++	12
Ever treated for TB before	15 (24%)
HIV-positive	1 (1.6%)
Body weight (kg)*	45 (29 – 63)
< 33 kg	6 (10%)
33-50 kg	39 (63%)
> 50 kg	17 (27%)
Medication (mg/kg body weight)*	
INH	7.31 (4.76 – 10.34)
rifampicin	11.97 (7.14 – 15.52)
pyrazinamide	36.58 (23.81 – 51.72)
ethambutol	18.29 (11.90 – 25.86)

* median (range)

From 62 patients, 97 samples were available for measurement of rifampicin. The mean time recorded between witnessed drug-intake and blood sampling was 2 hours (+/- 5 min). From 35 patients, values of two separate measurements were available. In these patients, the coefficient of variation (SD/n) of plasma rifampicin concentrations four, respectively eight weeks after start of treatment was 36%. Toxic concentrations (> 20 mg/L) of rifampicin were not detected in any of the 97 samples.

Two-hour plasma concentrations of rifampicin were within the therapeutic range in two patients (3%), low (4-8 mg/L) in 17 patients (27%), and very low (< 4 mg/L) in 43 patients (70%) (**Figure 1**). Three patients had undetectable rifampicin oncentrations. Rifampicin concentrations were significantly lower in male patients than in female patients (median 2.67 vs. 4.62 mg/L; *P* = 0.04; **Figure 1**). Fifty-three patients (85%) had been treated with drug formulations produced by manufacturer “A”, while 9 patients (15%) had been treated with drugs from manufacturer “B” (both are leading manufacurers in Indonesia). Plasma rifampicin concentrations were 2.35-fold higher (95% CI: 1.45 – 3.26; *P* = 0.001) in patients treated with drugs from manufacturer “B” than in patients treated with drugs from manufacturer “A”. No significant correlation was found between body weight and plasma concentrations of rifampicin (R^2 = 0.06). Diarrea (n=6) was not associated with lower plasma rifampicin concentrations. HIV-infection was established in one patient, whose two-hour plasma rifampicin concentrations were < 1 mg/L on two separate occasions.

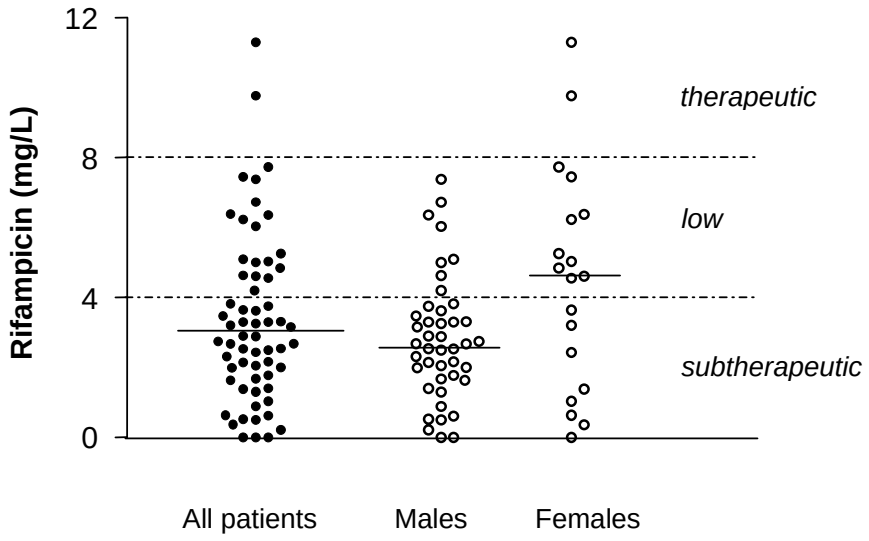

Figure 1.

Two-hour plasma rifampicin concentrations in 62 nonselected tuberculosis patients. Each dot represents one individual. Horizontal solid bars are the median for all patients, females and males respectively. Rifampicin concentrations > 8 mg/L were considered therapeutic, 4-8 mg/L low, and < 4 mg/L subtherapeutic.

rifampicin content of drug formulations.
From 59 patients, medication was available for analysis: 51 produced by manufacturer “A”, and eight by manufacturer “B”. Drug preparations from “A” were from three different batches. No medication was expired at time of treatment. The weight of tablets was 625 +/- 4.9 mg for “A”, and 901 +/- 18.9 mg for “B”. Rifampicin content was 93.6 +/- 1.3 % of the standard reference for tablets from “A”, and 100.1 +/- 2.4 % for tablets from “B”. No single preparation had a content $\geq$ 15% from the reference standard (range: 90.5 – 103.6%).

clinical and bacteriological significance of plasma rifampicin concentrations
During treatment, one patient died (1.6%) and 6 defaulted (9.7%). After six months treatment, 50 patients were cured (80.6%), two patients (3.2%) showed a bacteriological failure, and three patients (4.8%) were still on treatment because of delayed sputum conversion. The cure rate was higher in patients with rifampicin concentrations > 4 mg/L than in patients with concentrations < 4 mg/L (94.7% vs 74.4%), but this was mainly caused by a higher default rate in the latter group. Weight gain and resolution of symptoms were similar in both groups (data not shown). No jaundice or symptomatic hepatitis occurred during treatment. Three patients (4.8%) developed a mild elevation of plasma transaminases (twice the upper limit of normal); no patient had transaminases greater than three times the upper limit of normal. Plasma concentrations of rifampicin in these patients were 3.31, 3.76 and 7.38 mg/L respectively. After four weeks of treatment, concentrations of plasma rifampicin showed a weak correlation with plasma transaminases (R^2 = 0.10; *P* = 0.017).

The bacteriological response was similar in patients with therapeutic and subtherapeutic concentrations of rifampicin: after two months of treatment, sputum culture was positive for *M. tuberculosis* in 25% and 20% of patients with plasma rifampicin concentrations < 4mg/L and > 4 mg/L respectively. Minimal inhibitory concentrations (MIC’s) for rifampicin were available for 43 *M. tuberculosis* isolates. The MIC for rifampicin was > 1 mg/L in 2 patients (resistant; 4.6%), between 0,5 and 1 mg/L in 20 patients (intermediate; 46.5%), and < 0,5 mg/L in 21 patients (sensitive; 48.8%). The median ratio C_{max}/MIC for rifampicin was 9.7 (range 0 – 48.8). In 64% of patients with rifampicin concentrations < 4 mg/L, and in 16% of patients with concentrations > 4 mg/L, The C_{max}/MIC was < 10 (*P* = 0.018).

Discussion

In this Indonesian tuberculosis control clinic, 70% of patients had very low two-hour rifampicin plasma concentrations (< 4 mg/L), and no toxic concentrations of rifampicin were found. The absence of more than two-fold elevated serum transaminases during treatment, which normally occurs in 10-20% of cases, is in line with low exposure to antituberculous medication in this population.

Our study is not the first to report low plasma concentrations of rifampicin in tuberculosis patients. In HIV-infected patients, low or absent 2-hour plasma concentrations of rifampicin and reduced total drug exposure have been reported [7,18-20]. In 22 non-HIV-infected tuberculosis patients, selected for a slow clinical response, treatment failure or relapse, 14 (64%) had plasma concentrations of rifampicin below the two-hour target range of 8 mg/L [21]. HIV-infection (1.6% in this patient group), diabetes and gastric surgery, which may all result in delayed absorption of rifampicin [4], cannot explain our results. Since rifampicin is rather unstable, patient studies are vulnerable to artefacts. However, by using a cold-chain and stabilization by ascorbic acid we have circumvented breakdown of rifampicin. The distribution of the rifampicin concentrations found also argues against decay. To increase precision, the majority of patients was evaluated twice on two separate days. In light of biological variability, the variation between the two measurements is relatively low. Although drug dosages were not adjusted to body weight (in line with the national TB-program in Indonesia), this did not account for variation of rifampicin concentrations. In accordance with a previous study, rifampicin concentrations were significantly lower in male than in female patients [22].

Several recent reports have shown that drug content of antituberculous drugs may be insufficient [5,6]. Trade of “fake drugs” is widespread in some parts of the world [23]. Indonesia has had serious problems related to drug supply in recent years. We and others have occasionally found degraded and expired medication in Indonesia. However, no reduced rifampicin content was found in the tablets that had been prescribed to the patients in this study. Apart from drug content, other factors such as particle size, excipients and manufacturing process may affect bioavailability of rifampicin [24]. For example, major problems have been encountered in the manufacture of combined formulations of rifampicin plus INH and pyrazinamide [25]. In this study, more than a two-fold difference was found in plasma concentrations from patients treated with formulations from two different drug companies. Drug content of the respective formulations was only slightly different, which indicates that the problem must lie in reduced bioavailability. This finding reemphasizes the need for pharmacokinetic studies to ensure the quality of marketed formulations of a crucial drug like rifampicin [26].

The clinical significance of finding low plasma concentrations of antituberculous drugs remains unclear because of the size of this study, which was only designed to test for bioavailability. To evaluate therapeutic efficacy would require a much larger study, since under supervised treatment, the bacteriological failure rate is in the order of 1-2% [27,28]. However, several case reports suggest that treatment failure and acquisition of drug resistance occurs with low drug levels [7,18,21,29,30].

It is clear from the literature that the microbicidal effect of rifampicin is concentration dependent. The peak concentration after oral administration, which occurs around 2 hours after ingestion [14] should be between 8 and 20 mg/L. Rifampicin concentrations between 4 and 8 mg/L are in a gray zone, and concentrations below 4 mg/L are considered subtherapeutic. Such insights are derived from studies like the US Public Health Service trial in which a significantly higher percentage of bacteriological failures occurred in patients treated with 450 mg rifampicin than with 600 or 750 mg [8]. Patients in this trial treated with < 9 mg rifampicin per kg per day had a higher failure rate than those treated with > 9 mg / kg. In another study, a dose reduction of rifampicin from 600 mg to 300 mg significantly decreased bactericidal acitivity [31]. In line with a concentration dependent effect, less frequent administration of rifampicin has no detrimental effects [32]. Rifampicin requires a high ratio between maximum plasma concentrations in relation to MIC (high C_{max} / MIC) for optimal activity [17]. With a C_{max} > 8 mg/L and a normal MIC of 0.25 mg/L, the estimated C_{max} / MIC will be > 32. In our study, C_{max} / MIC was less than 10 in 50% of cases.

In summary, very low two-hour plasma rifampicin concentrations were found in the majority of a group of non-selected tuberculosis patients in Indonesia. The clinical significance of this finding is still unclear, but reduced bioavailability of rifampicin, and possibly other antituberculous drugs, may contribute to the low cure rates and frequent recurrence of tuberculosis in Indonesia [9]. Additional investigations are needed to evaluate the bioavailability of antituberculous medication and the pharmacokinetic properties of patients in this setting.

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Chapter 12

Summary and general discussion

After Robert Koch discovered *Mycobacterium tuberculosis* as the cause of TB in 1882, it would take more than 60 years until the first effective antituberculous drugs were developed. In 1948, in the first randomized controlled trial ever conducted [1], streptomycin showed remarkable results for treatment of TB. Soon thereafter it was discovered that mycobacteria readily become resistant to streptomycin alone. However, combination therapy with PAS, INH, and later rifampicin and pyrazinamide set the stage for eradication of TB. Now, another 60 years later, TB is more rampant than ever. Worldwide, millions of people, mostly in their productive age, suffer from active TB. Many of them die or become chronically ill. In large parts of the world, TB is as much the death sentence it was a hundred years ago.

TB is an intriguing disease. How does one explain the highly variable disease presentation and localisation ? What are the mechanisms behind latent TB and reactivation ? What is the pathophysiology of tissue necrosis in TB patients ? What is the nature of a protective host defense against TB ? Questions like these have been addressed for decades and are still relevant today. From a *pathophysiological* point of view, better understanding of susceptibility and protective immunity may contribute to development of more effective vaccines and new therapeutic strategies.

The *operational* approach has a very different starting point. Management of TB is complicated by problems of diagnosis and treatment. Late, false or missed diagnosis contribute to progression of TB and ongoing disease transmission. Inadequate or incomplete treatment and non-compliance of patients fuels drug resistance. Some of these issues are universal, but many problems are specific for particular geographic areas or health settings. Clinical, bacteriological and other (e.g. logistic) aspects of operational research may help to solve such problems. This will improve patient care and reduce the burden of tuberculosis.

The first half of this thesis contains studies dealing with *pathophysiological* issues related to host defense in TB. Cytokine studies in TB-patients raised questions about clinical and bacteriological aspects of TB, leading to investigations of operational problems. These studies constitute the second half of this thesis. Below is a summary of the content of this thesis.

Traditionally, acquired T-cell mediated immunity is thought responsible for protection in TB. Recently, attention has also focussed on natural, innate host defense mechanisms against *Mycobacterium tuberculosis*. **Chapter 2** is a review of such innate host defense mechanisms in TB. Our natural response to *M. tuberculosis* can be divided in phagocytosis, immune recognition, the inflammatory response, effector mechanisms, and initiation of the adaptive T-cell response. At each level, inter-individual differences may account for variable disease susceptibility and outcome. Several genetic polymorphisms that may code for these differences have

been identified. It is expected that growing understanding of disease pathogenesis will ultimately help design of adjunctive treatment, especially in drug- resistant TB.

Both innate and acquired immune responses are regulated by cytokines, the subject of study in **chapters 3-5**. The study of cytokine profiles in TB patients and control subjects may help to understand host defense in TB. Cytokine production in humans can be studied *in vivo*, in the circulation and at the tissue level, and *ex vivo* by stimulation of isolated cells. For *ex-vivo* cytokine production, whole blood assays have theoretical and practical advantages. In **chapter 3**, whole blood cultures were evaluated for non-specific and specific production of monocyte- and lymphocyte-derived cytokines. Dose-response, kinetics and variability of production of five pro- and anti-inflammatory cytokines were compared in undiluted and diluted whole blood. Stimulation and incubation of undiluted heparinized blood in closed vacutainer tubes has major advantages: simplicity, low contamination risk and minimal *in-vitro* manipulation. The addition of culture medium provides a more sensitive test using very small volumes of blood. PPD and MTB culture filtrate selectively induced production of interferon (IFN)-γ in skin-test positive individuals, and may therefore be used for diagnostic purposes and for identification and exploration of T-cell unresponsiveness in TB patients. The characteristics of whole blood determine both its advantages and drawbacks for cytokine assays: with optimal standardization, whole blood remains a ‘black box’ with many possible confounders. Still, this assay has been used successfully in several field studies, especially in Indonesia [2-4]. With slight adjustments, it is now employed in an immunogenetic study of TB in Indonesia [5].

In TB, cellular immunity is considered responsible for eradication of infection but also for damage of host tissues. In animal models, the balance between Th1-type cytokines, especially IFNγ, and Th2-type cytokines, primarily interleukin (IL)-4, seems crucial for these effects. There are conflicting reports on Th1-type and Th2-type cytokines in human tuberculosis, and little is known about their role in tissue damage. In **chapter 4**, flowcytometric assessment of cytokine responses in TB-patients and healthy controls in Indonesia revealed a similar production of IFNγ. In contrast, IL-4 production capacity of circulating T-lymphocytes from TB patients was markedly increased. Production of IL-4 was established in $CD4^+$ T-cells, the primary cytokine-producing cell in the Th1-Th2 concept, but also in $CD8^+$ T-cells. Interestingly, the expression of IL-4 was especially elevated in patients with cavitary tuberculosis. Therefore, as was previously shown in animal studies, these results suggest a role for IL-4 in the development of host tissue damage in human TB.

Tumor necrosis factor (TNF)-α is essential for defense against *M. tuberculosis*, but excessive production of TNFα or other proinflammatory cytokines may be responsible for deleterious effects in TB like fever, tissue necrosis and cachexia. Pentoxifylline and thalidomide, two drugs which inhibit TNFα production have shown some beneficial effects in mycobacterial infections in terms of decreased weight loss

and inflammation. Since these drugs have different mechanisms of action, combined therapy may lead to synergy, and thus may be advantageous in terms of toxicity and effectiveness. Conflicting reports have been published on the effects of these drugs on antiinflammatory cytokines. **Chapter 5** investigates *in-vitro* modulation of pro- and anti-inflammatory cytokines by either drug alone or in combination. Pentoxifylline more strongly inhibited *in-vitro* production of TNFα, while thalidomide more strongly inhibited IL-1β production. Both agents diminished release of IFNγ. No significant modulation of antiinflammatory cytokines IL-10 and IL-1Ra was achieved. When used together, pentoxifylline and thalidomide had additive, but no synergistic effects on the inhibition of TNFα and IFNγ. *In vivo* studies are needed to assess the possible role of combined use of these drugs in TB.

TB often leads to severe weight loss (wasting), probably through the production of inflammatory mediators. Wasting in turn, affects the inflammatory response, suppresses cellular immunity and aggravates disease outcome of TB. In these complex relations between TB, nutritional status and the host immune response, the adipocyte product leptin is a possible mediator. In **chapter 6** it is shown that plasma leptin concentrations were significantly suppressed in TB patients, more than could be explained by loss of body fat. Unexpectedly, leptin was inversely associated with C-reactive protein and TNFα production. These results argue against a causal role for leptin in TB-associated wasting. Rather, loss of body fat leads to low leptin concentrations, and prolonged inflammation may further suppress leptin production. As leptin is important for cell-mediated immunity, it may be concluded that low plasma leptin production during TB may contribute to increased disease severity, especially in cachectic patients.

The second half of this thesis consists of *operational* research and includes clinical and bacteriological studies. Under a microscope, *Mycobacterium tuberculosis*, which causes TB, cannot be distinguished from nontuberculous (atypical) mycobacteria, many of which are harmless. In clinical specimens, nontuberculous mycobacteria may represent true infection, colonization or contamination. In a Dutch TB clinic, diagnostic problems related to the presence of nontuberculous mycobacteria in some patients called for a retrospective analysis in this setting (**chapter 7**). It was shown that in this population, with a low HIV-prevalence, nontuberculous mycobacteria, although highly prevalent, poorly reflected true disease and often led to diagnostic and therapeutic errors. Although it is common knowledge that the presence of acid-fast bacilli is not specific for TB, it may be concluded that a higher index of suspicion is needed to circumvent this diagnostic pitfall. Close collaboration of microbiologists and clinicians and implementation of rapid DNA-based techniques for detection and typing of mycobacteria may improve clinical management.

Chapters 8-11 describe studies which were performed in Indonesia. The first cytokine study in TB in Indonesia in 1998 raised many clinical and bacteriological questions. Six months afterwards, in an urban TB clinic in Jakarta, a prospective clinical and bacteriological characterization of a cohort of patients was started (**chapter 8**). Patients in this setting presented with longstanding and severe disease. HIV-infection was uncommon. Malnutrition was highly prevalent and strongly related to mortality. Irrespective of previous treatment, resistance to antibiotics was commonly found among patient isolates. Unexpectedly, liver damage was completely absent during treatment. These observations generated new questions prompting further patient studies in Indonesia.

The yield of sputum microscopy in the Indonesian setting is addressed in **chapter 9**. In the first observational study in Jakarta, efforts had been made to increase the diagnostic rate. Introduction of larger sputum containers, and education of health personnel and patients resulted in more sputum samples of good quality. A sustainable two-fold rise of the smear-positive rate in this clinic was achieved. To further explore the role of education, a randomized study was conducted in another urban clinic. Compared with patients with suspected TB who underwent the routine procedure, those who received special instruction were smear-positive more often. Interestingly, education had a much larger effect in females. Simple and cheap interventions like these may help to increase the low case detection rate for TB in Indonesia. This may improve the outcome for the individual patient and may help to reduce transmission of TB.

Worldwide, DNA fingerprinting has revealed an extensive heterogeneity of *M. tuberculosis* genotypes. However, a distinct and predominant genotype, termed ‘Beijing’ has been found in recent years. Previous reports have shown that ‘Beijing’ strains are associated with (multi)-drug resistance. In **chapter 10** it is shown that strains of the ‘Beijing’-genotype are also present in the Indonesian archipelago. ‘Beijing’ strains examined showed a tendency towards higher drug resistance. Early in treatment, patients infected with ‘Beijing’ strains more often developed fever, unrelated to disease severity, toxicity or drug resistence. This suggests that specific pathogenic properties of ‘Beijing’ strains induce a different host response. Further study is needed to find out if this explains the high prevalence of this genotype.

Chapter 11 deals with treatment of TB in Indonesia. Two-hour plasma concentrations of rifampicin, a key drug for treatment of TB, were measured in TB patients. The large majority of patients showed sub-therapeutic concentrations. No toxic concentrations were found and liver damage, a common finding during TB treatment, was completely absent. A large difference was found in rifampicin concentrations from patients treated with drugs from two different manufacturers. Because the rifampicin content of the drug preparations in use was within the normal range, it can be concluded that bioavailability of locally produced drugs is substandard. This is currently examined in pharmacokinetic studies in Indonesia.

The link between *pathophysiological* and *operational* research in TB is a central aspect of this thesis. Pathophysiological (e.g genetic or immunological) patient studies very much depend on the quality of the clinical and bacteriological infrastructure. Operational projects not only improve research quality, but also generate study questions. For example, a first study describing the clinical and bacteriological status of a patient cohort in Jakarta led to hypotheses about BCG-vaccination, TB-associated wasting and lipid metabolism, and immunogenecits of different *M. tuberculosis* genotypes. Some of these hypotheses have now been tested.

In Indonesia, as in any country endemic for TB, there is yet another reason to conduct operational (i.e. *quality)* projects. First, TB research in high-endemic settings should address questions, which are relevant to that particular setting. In Indonesia, bacteriological, clinical and logistical projects will undoubtedly have more impact on TB-control than immunology and genetics. In large parts of the world, the situation is similar to that in Indonesia: limited resources to manage a large number of patients and questions related to the TB-program or to clinical management rather than to the pathogenesis of TB.

By themselves however, operational projects will not eradicate TB. Improved diagnosis and treatment will surely contribute to TB-control. But antibiotics are not the final answer, especially in light of growing drug resistance of *M. tuberculosis* isolates. A better understanding of the pathogenesis of TB is a first step towards the development of new strategies to fight TB. In recent years, basic science has produced exciting insights into TB, related to the mycobacterium as well as to the human host. However, many issues that troubled Robert Koch and other scientists a century ago, continue to trouble us today. Why are some individuals susceptible to TB, while others seem naturally protected ? What determines disease localization in TB ? What are the mechanisms behind latency and reactivation of TB ? What is the cause of TB-associated weight loss and tissue necrosis ? Renewed interest in TB provides the momentum, and modern technology the methods to tackle these issues. For TB as for any other health problem, the impact of scientific breakthroughs depends on the organization of the health system and quality of patient management. This reaffirms the need to combine a *pathophysiological* and *operational* approach.

This thesis and that of Elvina Karyadi [6] present the first TB studies we performed in Indonesia. In the past few years, a strong multidisciplinary team has been established concentrating on four different lines of patient-oriented research: two dealing with pathophysiological issues, and two with quality of care. The topics are (1) immunogenetic determinants of host defense in TB, (2) nutrition and metabolism in TB, (3) TB-diagnostics and (4) treatment of TB. First, immunogenetic markers of susceptibility and manifestations of tuberculosis are currently investigated in a large case-control study in Indonesia. Second, the mechanisms of wasting and

TB-associated changes in lipid- and carbohydrate metabolism will be examined. This may contribute to the design of nutritional and other interventions, which may benefit TB-patients in this setting. Our third focus is diagnostics. We are currently implementing a quality-controlled and optimized method for conventional drug susceptibility testing in Jakarta and Bandung. In addition, the feasibility of rapid (DNA-based) methods, e.g. for diagnosis of extrapulmonary TB will be examined in this setting. Fourth, we continue to evaluate and improve the quality and outcome of antibiotic treatment. Many contribute to this work, in Indonesia and the Netherlands. We hope that the various projects will add to increased understanding of the pathogenesis of TB, and will help to reduce the burden of TB in Indonesia and elsewhere.

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Nederlandse samenvatting

Nadat Robert Koch in 1882 had aangetoond dat *Mycobacterium tuberculosis* de verwekker was van tuberculose (TB), zou het nog meer dan 60 jaar duren tot er effectieve medicijnen tegen TB waren ontwikkeld. In 1948, in de eerste gecontroleerde klinische trial uit de medische geschiedenis, toonde streptomycine een uitgesproken effect bij de behandeling van TB. *M. tuberculosis* werd al snel resistent tegen monotherapie met streptomycine, maar combinatiebehandeling met PAS, INH en later rifampicine en pyrazinamide schiep de belofte voor eradicatie van TB. Nu, nog eens 60 jaar later, is TB nog altijd niet bedwongen. Wereldwijd lijden miljoenen, jong en oud, aan TB. Veel van hen komen te sterven of worden chronisch ziek. In grote delen van de wereld is TB dezelfde doodstijding als 100 jaar geleden.

TB is een intrigerende ziekte. Hoe verklaart men de zo diverse presentatie en localisatie van de ziekte ? Wat is het mechanisme achter latente TB en reactivatie ? Wat is de pathofysiologie van weefselnecrose in TB ? Waaruit bestaat een beschermende immuun- of gastheerrespons tegen TB ? Vragen zoals deze zijn nog even relevant als een eeuw geleden. Vanuit een *pathofysiologische* invalshoek zou een beter begrip van de vatbaarheid voor, en beschermende afweer tegen TB kunnen bijdragen tot ontwikkeling van effectievere vaccins en nieuwe therapeutische strategieën.

Tegenover de *pathofysiologische* staat een *operationele* benadering, welke zich richt op diagnostische en therapeutische problemen in de TB-zorg. Zo kan een late, incorrecte of gemiste diagnose leiden tot verergering van ziekte of onnodige verspreiding van TB. En zo voeden inadequate of incomplete behandeling het onstaan van antibioticaresistentie. Sommige van zulke operationele problemen zijn universeel, maar vaak zijn knelpunten specifiek voor een bepaald gebied of een bepaalde gezondheidsinstelling. Klinisch, bacteriologisch en ander (bijv. logistiek) gericht operationeel onderzoek kan helpen zulke problemen het hoofd te bieden. Dit zou de zorg voor TB-patiënten ten goede komen en verspreiding van TB remmen.

De eerste helft van dit proefschrift beschrijft *pathofysiologisch* onderzoek m.b.t. de gastheerrespons bij TB. Dit immunologisch onderzoek bij TB-patiënten riep klinische, bacteriologische en epidemiologische vragen op, welke leidden tot operationeel onderzoek. Het operationele onderzoek wordt beschreven in de tweede helft. Hieronder volgt een samenvatting van de inhoud van dit proefschrift.

Van oudsher wordt verworven cellulaire (T-cel) immuniteit verantwoordelijk geacht voor bescherming tegen TB. Recent bestaat er meer aandacht voor onze aangeboren afweer tegen *M. tuberculosis* (MTB). **Hoofdstuk 2** bevat een overzicht van zulke aangeboren afweermechanismen tegen TB. Onze aangeboren, natuurlijke, afweer tegen TB kan worden onderverdeeld in fagocytose, immuun herkenning, ontstekings-reacties, effector mechanismen en activering van de verworven cellulaire immuniteit. Op elk niveau kunnen interindividuele verschillen de variatie in

vatbaarheid en beloop van TB verklaren. Er zijn op DNA-niveau inmiddels een aantal subtiele afwijkingen (polymorfismen) gevonden welke zulke verschillen bij bepaalde patiënten kunnen verklaren. Het mag verwacht worden dat een groter begrip van de pathogenese uiteindelijk zal bijdragen tot ontwikkelen van adjuvante therapie, m.n. in multiresistente TB.

Zowel de aangeboren als de verworven immuniteit worden gereguleerd door kleine boodschappereiwitten, zgn. cytokines. Cytokineproductie kan bij patiënten *in vivo* worden gemeten, in de bloedsomloop en op weefselniveau, en *ex vivo* door stimulatie van geisoleerde ontstekingscellen. Voor het meten van de *ex-vivo* cytokineproductie heeft de 'volbloed-assay' theoretische en praktische voordelen. In **hoofdstuk 3** werden dosis-respons, kinetiek en variabiliteit van productie van vijf pro- en antiinflammatoire cytokines vergeleken in verdund en onverdund vol bloed. Stimulatie en incubatie van onverdund bloed in afgesloten heparinebuisjes heeft belangrijke voordelen in termen van eenvoud, risico op contaminatie en minimale in-vitro manipulatie. Toevoeging van kweekmedium levert een meer gevoelige assay met gebruik van minimale hoeveelheden bloed. Stimulatie van volbloed met eiwitbestanddelen van de mycobacterie geeft in volbloed-assays een selectieve inductie van interferon (IFN)-γ bij individuen met een positieve Mantoux-reactie en kan daarom gebruikt worden voor diagnostische doeleinden en voor het meten van specifieke T-celresponsiviteit bij TB-patienten. De kenmerken van volbloedassays bepalen zowel de voor- als de nadelen voor cytokine onderzoek. De volbloed assay leent zich bij uitstek voor veldstudies en is als zodanig met succes toegepast [2-5], maar ook met optimale standaardisatie blijft volbloed een 'black box' met veel mogelijke 'confounders'.

In TB wordt cel-gemediëerde immuniteit verantwoordelijk geacht voor eradicatie van infectie, maar ook voor de voor TB zo karakteristieke weefselschade. In deze lijkt in diermodellen de balans tussen zogenaamde Th1-type cytokines, m.n. IFNγ, en Th2-type cytokines, m.n. interleukin (IL)-4, van groot belang voor de uitkomst van mycobacteriële infecties. Tegenstrijdige bevindingen zijn gerapporteerd t.a.v. Th1-type en Th2-type cytokines bij patiënten met TB, en er is weinig bekend over hun rol bij weefselschade. In **hoofdstuk 4** werd m.b.v. flowcytometrie de cel-specifieke cytokinerespons vergeleken van TB-patiënten en gezonde controles: productie van IFNγ was gelijk, maar de capaciteit tot productie van IL-4 bleek sterk verhoogd bij TB-patiënten. Dit gold zowel voor CD4+ T-cellen, het primaire celtype van het Th1-Th2 concept, als voor CD8+ T-cellen. Interessant genoeg was de expressie van IL-4 met name verhoogd in patiënten met cavitaire (holtevormende) TB. Deze bevinding suggereert dat IL-4, in analogie met diermodellen, een rol speelt bij het ontstaan van weefselschade bij TB.

Tumor necrosis factor (TNF)-α is essentieel voor bescherming tegen TB, maar verhoogde productie van TNFα en andere proïnflammatoire cytokines lijkt verantwoordelijk voor schadelijke effecten in TB als koorts, weefselnecrose en

cachexie. Gebruik van pentoxifylline and thalidomide, twee medicamenten die de productie van TNFα remmen, hebben bij mycobacteriële infecties geleid tot afname van gewichtsverlies en ontsteking. Daar deze stoffen een verschillend aangrijpingspunt hebben, zou gecombineerde behandeling synergistisch kunnen werken, en zo meer effectief of minder toxisch kunnen zijn. Er bestaat in de literatuur geen eenduidigheid wat betreft het effect van pentoxifylline and thalidomide op antiinflammatoire cytokines. **Hoofdstuk 5** beschrijft de *in-vitro* modulatie van pro-en antiinflammatoire cytokines van pentoxifylline and thalidomide apart en tezamen. TNFα werd krachtiger geremd door pentoxifylline, en IL-1β door thalidomide. Beide stoffen remden de in-vitro productie van IFNγ. Er werd geen significante modulatie gevonden van productie van antiinflammatoire cytokines IL-10 en IL-1Ra. Tezamen hadden pentoxifylline and thalidomide een additief maar geen synergistisch effect op remming van TNFα en IFNγ. *In vivo* studies zijn noodzakelijk om de eventele plaats van deze middelen nader te bepalen voor de behandeling van TB.

Zoals boven opgemerkt gaat TB regelmatig gepaard met ernstig gewichtsverlies. Omgekeerd onderdrukt ondervoeding de menselijke afweer tegen TB en leidt zij tot een ongunstiger ziektebeloop. Leptine, een door vetcellen geproduceerd eiwit, is een mogelijke mediator in deze complexe interactie tussen TB, voedingsstatus en de gastheerrespons. In **hoofdstuk 6** wordt beschreven hoe plasma leptine concentraties substantieel verlaagd bleken bij TB-patiënten in Indonesië, meer dan op grond van verlies van vetmassa kon worden verklaard. Onverwachts toonde leptine een omgekeerd verband met twee ontstekingsmediatoren: C-reactive protein en TNFα. Deze resultaten pleiten tegen een oorzakelijke rol voor leptine in TB-geassocieerd gewichtsverlies. Het lijkt waarschijnlijker dat verlies van vetmassa bij TB-patiënten leidt tot verlaagde leptine-waarden, en dat ontsteking de leptineproductie verder onderdrukt. Leptine is belangrijk voor T-cel-immuniteit. Mogelijk dragen lage leptine-spiegels bij tot een slechte afloop van TB, vooral bij sterk vermagerde patiënten.

De tweede helft van dit proefschrift beschrijft *operationeel* onderzoek en omvat klinische en bacteriologische studies. Onder de microscoop is *Mycobacterium tuberculosis*, de verwekker van TB, als zuur-vaste staaf niet te onderscheiden van nontuberculeuze (atypische) mycobacteriën, waarvan vele onschuldig zijn. In patiëntenmateriaal kunnen nontuberculeuze mycobacteriën wijzen op echte infectie, maar ook op kolonisatie of contaminatie. In een Nederlandse TB-kliniek hadden diagnostische problemen gerelateerd aan het voorkomen van nontuberculeuze mycobacteriën bij enkele patiënten geleid tot het doen van een retrospectief onderzoek (**hoofdstuk 7**). Het bleek dat nontuberculeuze mycobacteriën in de bewuste populatie weliswaar vaak werden aangetroffen, maar slechts zelden duidden op werkelijke infectie. E.e.a. had regelmatig geleid tot diagnostische en therapeutische fouten. Alhoewel het algemeen bekend is dat de aanwezigheid van

zuur-vaste staven in patiëntenmateriaal niet specifiek is voor TB, kan geconcludeerd worden dat een grotere alertheid gewenst is om deze diagnostische valkuil te ontwijken. Nauwe samenwerking tussen microbiologen en klinici en toepassing van betrouwbare moleculair-biologische methoden voor het opsporen en typeren van mycobacteriën zullen het klinische beleid doen verbeteren.

Hoofdstukken 8-11 beschrijven onderzoek verricht in Indonesië. Het eerste onderzoek (over cytokines) in Indonesië in 1998, beschreven in hoofdstuk 4, riep veel vragen op over de diagnostiek en behandeling van TB aldaar. Zes maanden later ging in een kliniek in een arme wijk in centraal Jakarta een prospectief klinisch-bacteriologisch, observationeel onderzoek van start (**hoofdstuk 8**). Patiënten in deze setting presenteren zich met langbestaande en ernstige longtuberculose. HIV-infectie was zeldzaam, maar ondervoeding was frequent aanwezig en sterk geassocieerd met sterfte. Onafhankelijk van eerdere behandeling bleek een belangrijk deel van de patiënten besmet met resistente TB-bacillen. Deze waarnemingen leidden tot nieuwe vragen en meer patiëntgebonden onderzoek in Indonesië.

De opbrengst van sputum microscopie voor TB in de Indonesische setting werd geëvalueerd in **hoofdstuk 9**. Tijdens de eerste observationele studie in Jakarta was getracht de diagnostische opbrengst van sputumonderzoek te vergroten. Grotere sputumpotten en voorlichting voor medewerkers en patiënten in de kliniek leidden tot meer sputum monsters van goede kwaliteit. Een blijvende verdubbeling van ZN-positiviteit werd bereikt. Om de rol van instructie verder te onderzoeken werd in een andere kliniek een gerandomiseerd onderzoek uitgevoerd. Vergeleken met patiënten verdacht voor TB die de routine procedure ondergingen, waren diegenen die extra voorlichting over belang van sputumonderzoek en techniek van ophoesten hadden gekregen, vaker positief. Opvallend genoeg was het effect veel groter bij vrouwelijke patiënten. Simpele en goedkope interventies zoals deze kunnen helpen de lage case-detection rate voor TB in Indonesië te verhogen. Dit komt de uitkomst voor de individuele patiënt ten goede en zal bijdragen om verspreiding van TB terug te dringen.

Genotypering (‘DNA-fingerprinting’) van tuberculose-stammen laat werldwijd een enorme genetische diversiteit zien, maar recent is een predominant genotype gevonden, genaamd “*Beijing*”. Stammen van het *Beijing*-genotype bleken veelal geassocieerd met antibioticaresistentie. In **hoofdstuk 10** wordt aangetoond dat stammen van het *Beijing*-genotype ook in Indonesië voorkomen, en inderdaad vaker drug-resistent zijn. Opvallend genoeg bleek dat patiënten geïnfecteerd met *Beijing* stammen tijdens behandeling veel vaker koorts ontwikkelden, onafhankelijk van ernst van ziekte, drug-resistentie of toxiciteit. Dit suggereert dat *Beijing*-stammen bepaalde eigenschappen hebben welke een andere gastheerrespons induceren. Inmiddels bestaat hiervoor ook bewijs in verschillende diermodellen.

Hoofdstuk 11 behandelt een aspect van de behandeling van TB in Indonesië. Plasma-concentraties van rifampicine, één van de belangrijkste anti-TB middelen,

werden gemeten twee uur na inname van de dagelijkse medicatie. Ruim 70% van de TB-patiënten had sterk verlaagde rifampicine concentraties. Schadelijke rifampicine spiegels werden niet gevonden, net zo min als (asymptomatische) hepatotoxiciteit. Er was een twee-voudig verschil in rifampicineconcentratie tussen patiënten behandeld met antibiotica van twee verschillende lokale fabrikanten. Het rifampicinegehalte van verzamelde tabletten was normaal en derhalve lijkt de biobeschikbaarheid van lokaal geproduceerde medicatie onvoldoende. Op dit moment wordt de farmacokinetiek van antibiotica van vier fabrikanten vergeleken in gezonde Indonesische vrijwilligers.

De link tussen *pathofysiologisch* en *operationeel* onderzoek in TB vormt de rode draad van dit proefschrift. Pathofysiologisch (bijv. genetisch of immunologisch) onderzoek bij TB-patiënten valt en staat met de kwaliteit van de klinische en bacteriologische infrastructuur. Operationele projecten verbeteren zo de kwaliteit van onderzoeksgegevens, maar leveren ook nieuwe vragen voor pathofysiologisch onderzoek. Zo leidde de eerste beschrijving van de klinisch-bacteriologische status van een groep patiënten tot hypothesen over BCG-vaccinatie, gewichtsverlies en vetmetabolisme bij TB, en pathofysiologische eigenschappen van verschillende *M. tuberculosis* genotypen. Sommige van deze hypothesen zijn inmiddels getoetst in nieuwe projecten.

In Indonesië, zoals in de elk land waar TB een groot probleem is, bestaat er nòg een reden om operationele (lees: *kwaliteits*-) projekten uit te voeren. In dergelijke landen hebben bacteriologische, klinische en logistieke projekten nameliijk zonder twijfel meer effect op TB-controle dan immunologie en genetica. In grote delen van de wereld is de situatie niet anders. Met beperkte middelen moet voor enorme aantallen TB-patiënten worden gezorgd. TB-onderzoek in die landen zou zich primair moeten bezig houden met (voor daar) relevante onderzoeksvragen betreffende organisatie en kwaliteit van zorg.

Het is echter onwaarschijnlijk dat operationeel onderzoek alléén tot eradicatie van TB zal leiden. Verbetering van diagnose en behandeling zullen ongetwijfeld bijdragen aan bestrijding van TB. Maar antibiotica vormen niet het uiteindelijke antwoord, vooral in het licht van toenemende antibiotica-resistentie van TB-bacillen. Een beter begrip van de pathogenese van TB is een eerste stap op weg naar het ontwikkelen van nieuwe strategieën om TB te bestrijden. Recent basaal onderzoek heeft nieuwe inzichten verschaft, zowel met betrekking tot de tuberkelbacil als tot de TB-patiënt. Toch zijn veel vragen die Robert Koch en andere wetenschapers zichzelf een eeuw geleden stelden vandaag nog steeds niet goed opgelost. Waarom zijn sommige individuen vatbaar voor TB, terwijl anderen van nature beschermd lijken ? Wat bepaalt dat de plaats van de TB-infectie in het lichaam ? Wat is het mechanisme van latente infectie en reactivatie van TB ? Wat is de oorzaak van het gewichtsverlies en de necrose (weefselschade) bij TB ? Hernieuwde interesse in TB levert het momentum, en moderne technologie de methoden om deze problemen te doorgronden. Voor TB, zoals voor elk gezondheidsprobleem, hangt de impact van

wetenschappelijke doorbraken op de dagelijkse praktijk af van de organisatie van het gezondheidssyteem en de kwaliteit van patiëntenzorg. Dit onderstreept nogmaals het belang om *pathopfysiologisch* en *operationeel* onderzoek te blijven combineren.

Dit proefschrift en dat van Elvina Karyadi [6] presenteren het begin van een onderzoekslijn op het gebied van TB in Indonesië. In de afgelopen jaren heeft zich inmiddels een sterk multidisciplinair team gevormd dat zich concentreert op vier verschillende lijnen van patiënt-gebonden onderzoek: twee over de pathofysiologie van TB, en twee over kwaliteit van zorg. De onderwerpen zijn: (1) immunogenetische determinanten van de afweer tegen TB, (2) voeding en metabolisme in TB, (3) bacteriologische diagnostiek van TB en (4) behandeling van TB. Allereerst worden markers van gevoeligheid voor infectie en presentatie van TB op dit moment onderzocht in een grote case-controle studie in Indonesië. Ten tweede worden de mechanismen van gewichtsverlies en veranderingen van koolhydraat en vet-metabolisme bij TB onderzocht. Dit kan mogelijk bijdragen tot het ontwikkelen van voedingssupplementen of andere interventies welke de TB-patiënten in deze setting ten goede kunnen komen. Ons derde aandachtspunt is de diagnostiek. Op dit moment wordt een simpele doch uiterst preciese methode voor conventionele bepaling van antibioticaresistentie in Jakarta en Bandung geïmplementeerd. Daarnaast wordt de haalbaarheid en de waarde van snelle (moleculair-biologische) methoden nagegaan, o.a. voor het opsporen van TB buiten de longen. Ten vierde evalueren en verbeteren we continu de kwaliteit en uitkomst van antibiotische behandleling. Velen dragen bij aan dit werk, in Indonesië en in Nederland. Wij hopen dat de uiteenlopende projecten het begrip van de pathogenese van TB zullen vergroten, en zullen helpen om de omvang en de ernst van TB te verminderen in Indonesië en daarbuiten.

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Curriculum vitae

Reinout van Crevel, de schrijver van dit proefschrift, werd geboren op 1 mei 1967 in Rotterdam. In 1985 deed hij zijn eindexamen aan het Vossius Gymnasium, Amsterdam. Tussen 1986 en 1994 volgde hij zijn medische opleiding aan de Universiteit van Amsterdam. In het eerste jaar van zijn studie verbleef hij in het kader van een verpleeghulpstage in een missieziekenhuis in Ghana. Tijdens zijn derde jaar volbracht hij een wetenschappelijke stage aan de Universiteit in Cambridge, en voor zijn co-schappen deed hij klinische ervaring op in Zimbabwe. Na het behalen van zijn doctoraal was hij van april 1994 tot januari 1995 werkzaam op de Afdeling Interne geneeskunde van het Onze Lieve Vrouwe Gasthuis in Amsterdam (opleider: Dr. B. Silberbusch). Vanaf januari 1995 is hij in opleiding tot internist aan het Universitair Medisch Centrum St Radboud, Nijmegen (opleider: Prof. Dr. J.W.M. van der Meer). Vanaf 1997 heeft hij in het kader van een NWO-stipendium Infectieziekten zijn opleiding gecombineerd met promotieonderzoek op het gebied van tuberculose (TB). Vanaf april 1998 heeft hij in dat kader in toenemende mate samengewerkt met klinici en bacteriologen in Jakarta en Bandung, Indonesië. Sindsdien coördineert hij daar verschillende kwaliteitsprojecten op het gebied van TB. Vanaf september 2000 is hij mede verantwoordelijk voor een KNAW-project in Jakarta op het gebied van immunologische en genetische factoren betrokken bij de afweer tegen TB. Vanaf november 2001 heeft hij zijn opleiding vervolgd in het Canisius Wilhelmina Ziekenhuis, Nijmegen (opleider: Dr. R.W. de Koning).
Hij is gehuwd met Denise Telgt en heeft twee dochtertjes, Rosa Luna en Isabel.